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Biocatalytic oxidase: Batch to continuous

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ABSTRACT

The adoption of more efficient development strategies and manufacturing techniques will be essential for future success in the bio manufacturing sectors. Continuous operation of biocatalytic processes has the potential to offer many advantages over established batch process methodologies. There exist opportunities for improved process control; ease of scale up; minimizing of interruptions in production; reducing reactor size; and economic use of biocatalysts.

The Coflore™ Agitated Cell Reactor (ACR) is a dynamically mixed plug flow reactor. The Coflore design employs a patented mixing technique where free moving agitators within each reaction stage promote mixing when the reactor body is subjected to lateral shaking. Multiple discrete (interlinked) reaction cells give good mixing and plug flow, and the design permits the use of slurries and handling of gas/liquid mixtures. The Coflore Agitated Tube Reactor (ATR) is an industrial tube flow reactor for homogenous and two phase fluids. Employing the same mixing principle as the lab scale Coflore ACR, it uses lateral movement to generate mixing and stage separation to prevent back mixing.

We describe the application of these continuous plug flow reactors for bioprocess development starting from simple lab scale batch processes; through benchtop plug flow reactors (ACRs); and on to the multi-litre production scale agitated tube reactor (ATR). The presentation will compare the results of an oxidation reaction catalysed by D-amino acid oxidase (DAAO) operated under batch and continuous conditions, and will illustrate how application of the ACR and ATR reactors can facilitate process development.

Keywords: Biocatalysis; Continuous flow; BIOCHEMIST; Coflore; Oxidase; Multiphase

1. Introduction

With a strong trend for automation in pharmaceutical research, high-throughput chemistry is still carried out in batches; whereas flow-through processes are restricted to production processes (Jas and Kirschning, 2003). Unlike batch reactors, the output of a flow device can be changed without altering the hardware or set-up conditions. This flexibility saves time and cost in development. The main advantages of the continuous approach are facile automation, reproducibility, safety and process reliability as reaction parameters are more easily controlled. The improved control capabilities of flow systems can also deliver better yield and productivity (Fig. 1). A fully optimised flow process can be used to continually synthesise complex products in a single process from inexpensive and simple starting materials, a task unparalleled by batch chemistry methods (Bartrum et al., 2010; Baxendale et al., 2006; Bogdan et al., 2009; Benito-Lopez et al., 2008).

Traditionally, flow systems have been considered unable to handle multiphase systems and long reaction times efficiently. Continuous flow processes are complemented by current trends in modern synthetic chemistry as they can be performed using immobilised catalysts or reagents (Ley and Baxendale, 2002; Drewry and Coe, 1999). Many continuous-flow processes are already established in synthetic chemistry (Ley et al., 2006), however, the uses of flow reactors in bio applications are still limited with only a handful reported in the literature (Coughlin et al., 1975).

The use of microreactors, Mason et al. (2007) has been widely reported in academia and yet only relatively recently has it been reported describing their industrial use (Markowz et al., 2005). In these types of reactor laminar flow dominates

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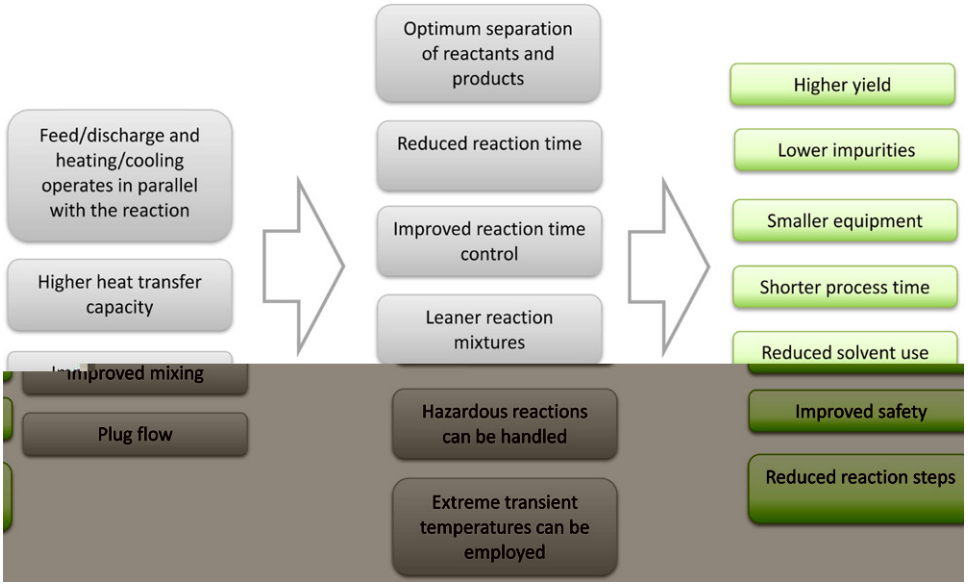

Fig. 1 – Showing how the characteristics of a continuous flow reactor can affect the process parameters.

and they use diffusion to mix the reactants in a very small channel (Sudarsan and Ugaz, 2006). They also exhibit excellent plug flow at low Reynolds numbers allowing a large array of conditions to be trialled in a very short space of time (Wiles and Watts, 2008). Of course these types of reactors are limited by scale but the advantages they show are crucial to the success of process flow chemistry. The work reported here will use these advantages to scale up processes to pre-production scale.

Three UK technology companies, C-Tech Innovation, Ingenza and AM Technology are collaborating to develop new flow process techniques for bio manufacturing. Co-funded by the Technology Strategy Board, the initial phase of the BIOCHEMIST project will run for two years. The project will integrate all aspects of bioprocess development from catalyst discovery and engineering, to process design, through to small footprint manufacturing of high value products.

The main focus of the project is a comparison between a batch process and a continuous plug flow process. A simple chiral resolution is used as the test reaction comparing a 1 L tank reaction with a Coflore ACR (agitated cell reactor) and a Coflore ATR (agitated tube reactor) reactor. BIOCHEMIST provides an opportunity to make radical changes in the reactor format necessary to accommodate the special features of biocatalytic processes, e.g. the need for process intensification to radically improve space time yields, develop units which can accommodate free and immobilized enzymes/cells in formats which can improve biocatalyst performance (including extending T1/2), and establish continuous processes which are not plagued by the problems of pressure drop, poor mass transfer and catalyst carrier attrition associated with many column-based continuous reactors. The reactor modification and redesign and trials proposed in the project will put convenient, effective and flexible process development scale up and manufacturing tools at the disposal of chemists which will condense the process development time and help establish highly efficient continuous biocatalytic processes.

2. Process principle

DL-Amino acid is resolved biocatalytically by oxidation of the D-amino acid giving a mixture of L-amino acid and the α-ketoacid using wild-type D-amino acid oxidase (Fig. 2). The biocatalyst is produced by fermentation of *Pichia pastoris* expressing the DAAO enzyme. The whole cells from the fermentation are freeze-dried and added to the biotransformation vessel. Oxygen is required as co-substrate and is added to the reaction via a sparged gas inlet. Initially, the reactions are usually run oxygen limited due to the gas–liquid mass transfer constraints of the vessels used. The reactions are monitored by HPLC and are deemed to be complete when the enantiomeric excess (ee) of the L-amino acid is >99%.

In the case of the amino acid oxidase, a metal catalysed reduction is combined with the oxidase biocatalyst to effect deracemisation of a racemic starting material (or stereoinversion of the single opposite enantiomer) to produce the target product in high optical purity. The use of the co-reagents in the bioprocess introduces additional parameters which, using conventional stirred tank reactor vessels, increases the complexity and time to develop and optimize the process towards each target. A by-product of the reaction is hydrogen peroxide which can affect the reaction by: (a) causing decomposition of the biocatalyst and (b) reacting with the keto-acid product giving rise to the C-1 carboxylic acid (decarboxylation, e.g. pyruvic acid to acetic acid).

Therefore a major innovation in this project is the combination of the highly adaptable and controllable Coflore reactor with the bioprocess which are fundamentally capable

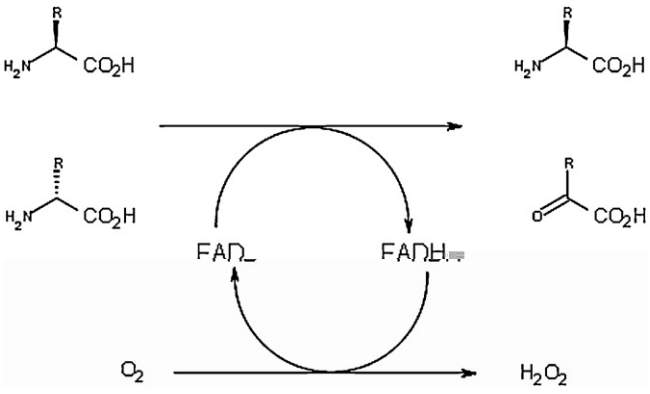

Fig. 2 – Biocatalytic resolution of DL-alanine.

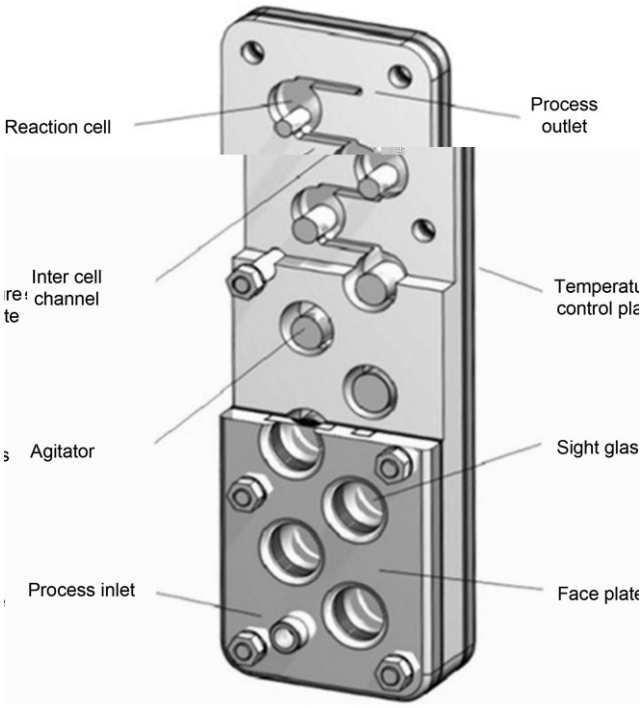

Fig. 3 – Coflore ACR.

of highly efficient and cost-competitive operation but which might otherwise have development times which are excessive for the aggressive timelines of pharmaceutical development.

3. The Coflore agitated cell reactor

The Coflore ACR is a multistage flow reactor initially designed for lab development and small scale manufacturing based on conventional chemical processes. The core of the ACR is a PTFE block with 10 equal sized holes interconnected by grooved channels (Fig. 3). The reactor is designed so to take advantage of the benefits that continuous flow offers over batch processes (Solodenko et al., 2007).

A heat transfer plate and 10 glass windows to monitor each cell are incorporated in the design. Inside each cell, agitating rollers can be placed and they may carry different functions, such as offering flexible cell volume, ensure consistent mixing, accommodate fluids of varying viscosity and provide containment for catalysts. The reactor block is mounted on an agitating platform which causes the rollers to move and the

Fig. 4 – ACR mounted in the shaking platform.

Fig. 5 – Gas/liquid phase inside ACR.

agitation can be varied in intensity in order to achieve from mild to vigorous shaking (Fig. 4). The simple internal geometry and strong mixing throughout the Coflore reactors provides an effective means of keeping multiphase mixtures suspended and well dispersed, to maintain orderly flow through the reactor.

The entire unit has a small footprint—making it suitable for using on the standard research laboratory bench top or fume cupboard as appropriate. Initial trials of the reactor have focused on understanding reactants' physical behaviours such as mixing, throughput of slurries and performance of 3-phase reactions. Results from these trials have highlighted actual improvements in process efficiency (increased yields, improved mixing, and efficient reagent use—as compared to conventional stirred tank systems). In this study, resolution of a simple amino acid is used to compare the process conditions between a batch reactor and the Coflore ACR. Product flows through a series of ten stirred cells under temperature controlled conditions. The dynamic mixing allows testing of different residence times, up to a few hours, at the lab scale size and with no detrimental effect on the mixing and plug flow capabilities. Oxygen is injected through the front of the first cell and flows upwards to the exit. The mixing is sufficient to achieve fine gas dispersion in the liquid allowing a good mass transfer and fast reaction time (Fig. 5). The most problematic issue for standard continuous flow reactors is fouling due to solid build up occurring at back pressure regulators and small gauge tubing connectors or at sharp turns in the reactors channelling (Ley, 2010). All these are present in the Coflore design but up to now minimal levels of fouling have been observed.

The agitators move in rapidly reversing transverse movements and in turn generate efficient mixing without the need for baffles. By employing this transverse mixing method as opposed to conventional rotational mixing, the problems of

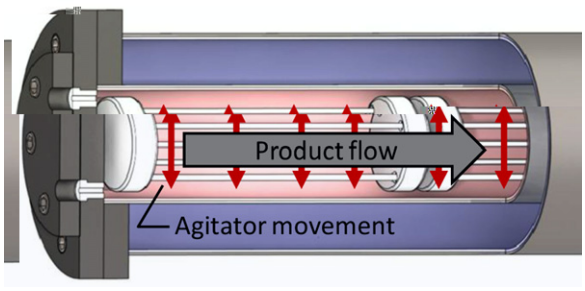

Fig. 6 – The ATR is a tubular reactor which uses the same mixing technique as the ACR. Capacity: 0.25–10 L.

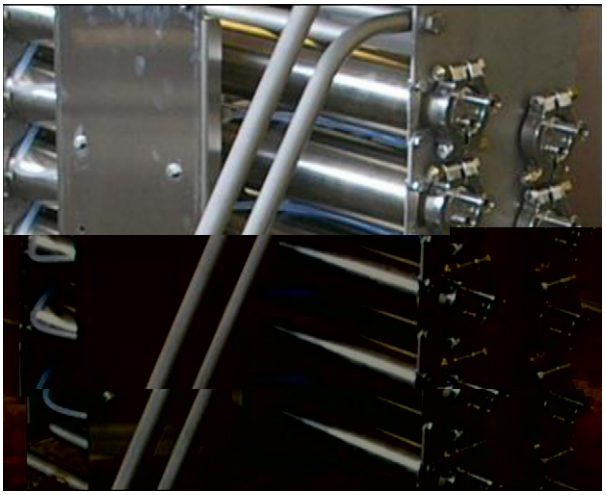

Fig. 7 – Industrial Coflore ATR platform.

centrifugal separation are avoided when materials of different density are present. The agitators do not use drive shafts for motive power which negates the requirement for further mechanical seals or magnetic couplings and avoids the problems associated with seal leaks, buffer fluids and stabilizing bushes. With regard to the processing of slurries, the current available reactors are geared to specific process problems and not a general solution (Takagi et al., 2004). The Coflore ACR is very simple in design with no dead volumes to cause solid build up. This particular mode of mixing is ideal for keeping suspensions uniformly dispersed and preventing solids from settling out.

4. The Coflore™ agitated tube reactor

The Coflore ATR is a tubular reactor with loose agitating elements (Fig. 6). By increasing the length of the reaction cells, process critical parameters (plug flow, mixing and heat transfer) remain substantially unchanged when scaled up from lab to industrial scale.

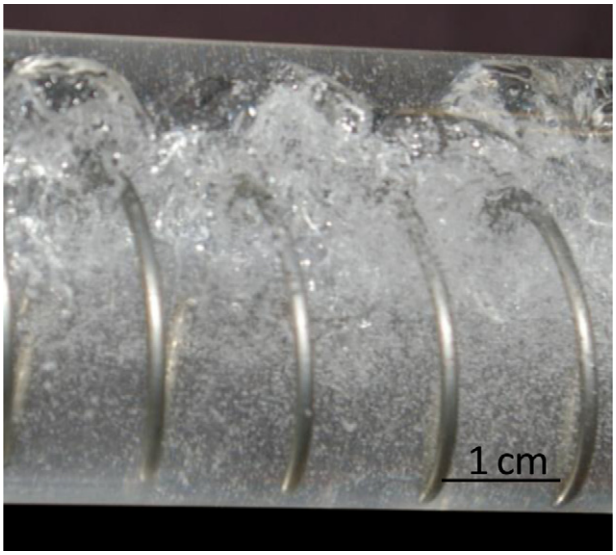

Fig. 8 – Viewed from side A high gas fraction with a large diameter spring operating at a high mixing speed. Increased gas concentration results in higher mixing intensity to create a good dispersion of small gas bubbles.

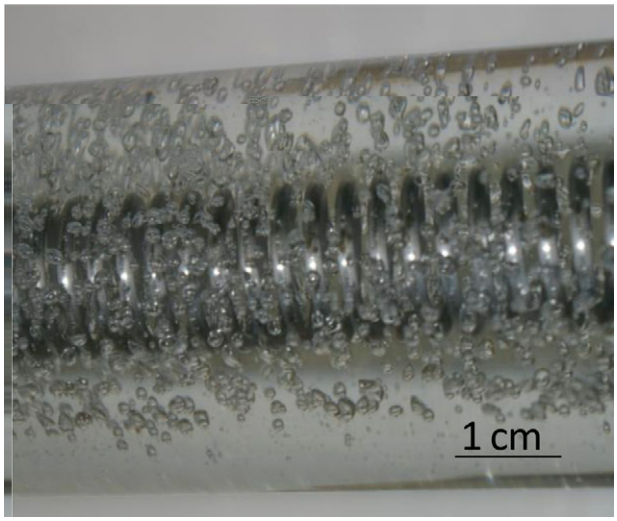

Fig. 9 – Viewed from above. A small diameter spring operating at a moderate speed. This produces uniformly sized gas bubbles and good dispersion.

The reactor body is mounted on a shaking platform and consists of a series of jacketed reactor tubes. Loose agitator elements generate strong radial mixing when the reactor body is shaken. The design of the agitators can be varied to achieve good mixing and adapt the surface to volume ratio (up to 1000 m^2/m^3).

The prototype Coflore ATR is a 10 stage reactor with a capacity of 10 L and a total length of 8 m (Fig. 7). The initial mixing tests were performed with a 40 mm glass reactor tube (for good visibility) using simple springs as mixers. The purpose of this exercise was to observe the distribution of an air/water mixture under agitated conditions. The reactor tube is mounted in a horizontal position and the photographs (Figs. 8 and 9) show the dispersion pattern of gas bubbles in the reactor tube under different operating conditions.

5. Experimental results

5.1. *Batch reactor*

The system under test is a resolution of DL-alanine to produce a mixture of L-amino acid and a-ketoacid using non immobilized enzymes on whole cells. Oxygen is required as a co-substrate and is added into the reaction through a sparged gas inlet. Reactions are carried out at a concentration of 1 mol alanine (89.09 g) per litre of water. All the experiments carried out were completed in duplicate and the results are averaged. If large differences were seen between results then the individual experiments were repeated.

Fig. 10 shows the progress of the reaction in a 1 L batch reactor with two different catalyst loadings. As results show, catalyst loading has negligible effect on reaction rate under these conditions.

In Fig. 11, the agitator speed is varied in the 1 L batch vessel. The results show a substantial increase in reaction rate as the agitator speed is increased. After about 5 h however the reaction seems to fall to a substantially lower and constant rate.

The initial improved reaction rate at higher agitation speeds can be attributed to improved oxygen uptake associated with more efficient mixing. The slower and relatively constant rate after 5 h can be explained by reaction shifting

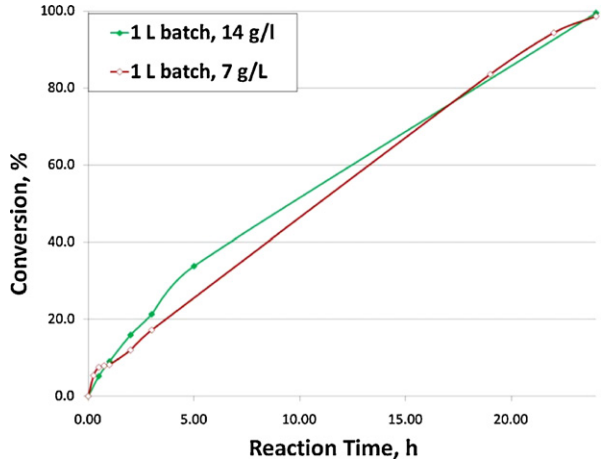

Fig. 10 – Effect of enzyme loading in a 1 L batch reactor. Stirrer speed = 400 rpm, oxygen flow = 1 L/min.

from oxygen uptake limited to enzyme limited, due to the depletion of enzyme.

The reaction was also performed in a smaller 250 mL batch reactor and gave higher reaction rates than the 1 L vessel for comparable agitator speeds. This can be attributed to the improved mixing due to smaller vessel size. Further scale up was carried out at 4 L (Fig. 12). A largely decelerated reaction rate can be seen as the process is scaled up. It is thought that this problem will increase with increasing vessel size due to the lower mixing efficiency at larger scale.

5.2. *Flow conditions*

This reaction was repeated under flow conditions. The Coflore ACR reactor was used for this as it provides good gas/liquid mixing and tolerates the presence of solids.

Fig. 13 shows a slightly increased reaction rate in the lab scale ACR as opposed to the 250 mL batch reactor. The higher performance of the ACR can be attributed to its small diameter and use of transverse mixing (which is self baffling and does not promote centrifugal separation). The effect seen here is also seen when comparing a 1 L batch with a 1 L tube on the ATR (Fig. 14). The ACR results show a 'plateau' effect around 2.5 h, an effect not seen in the ATR. Further experiments are currently underway to investigate the stability of

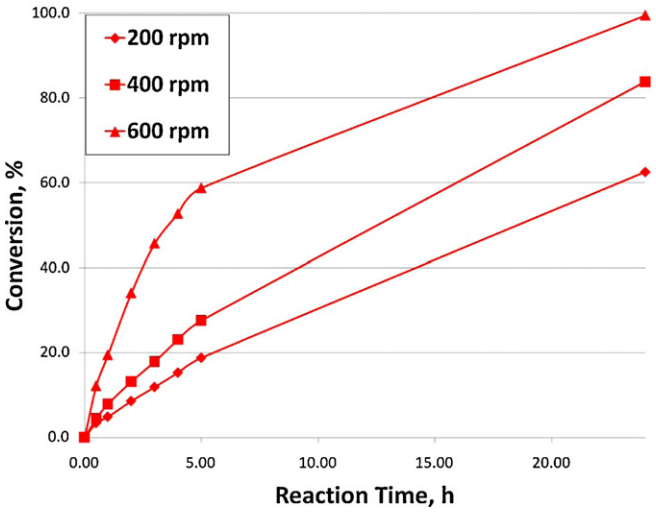

Fig. 11 – Effect of stirrer speed in a 1 L batch reactor. Enzyme load = 7 g/L, oxygen flow = 0.625 L/min.

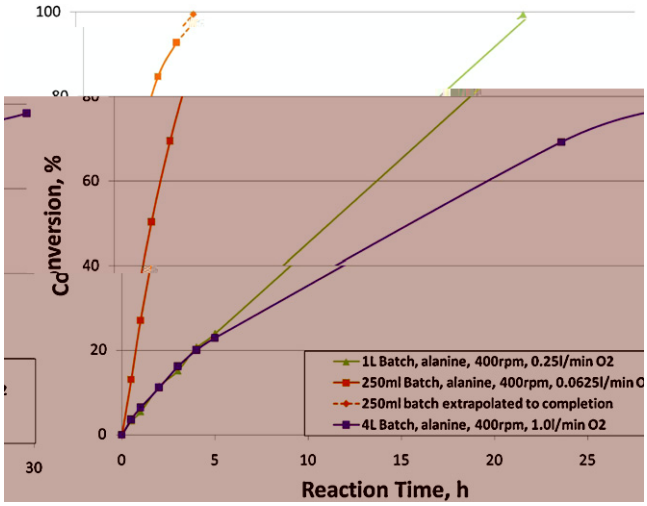

Fig. 12 – Scale up of batch process. Enzyme load = 21 g/L, stirrer = 400 rpm for batch. Oxygen: as shown in graph legend.

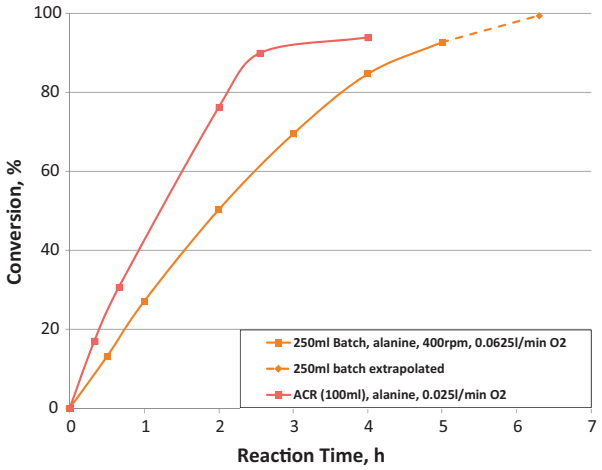

Fig. 13 – Comparison between flow and batch process. Enzyme load = 21 g/L, stirrer = 400 rpm for batch. Oxygen: 0.0625 L/min batch, 0.025 L/min ACR.

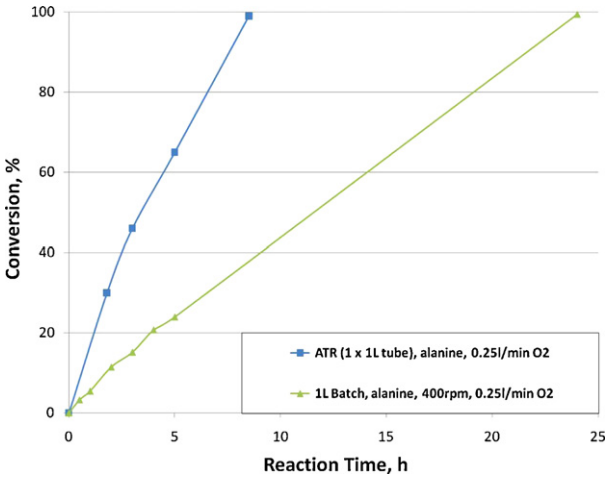

Fig. 14 – Comparison between flow and batch process. Enzyme load = 21 g/L, stirrer = 400 rpm for batch. Oxygen: 0.25 L/min batch, 0.25 L/min ATR.

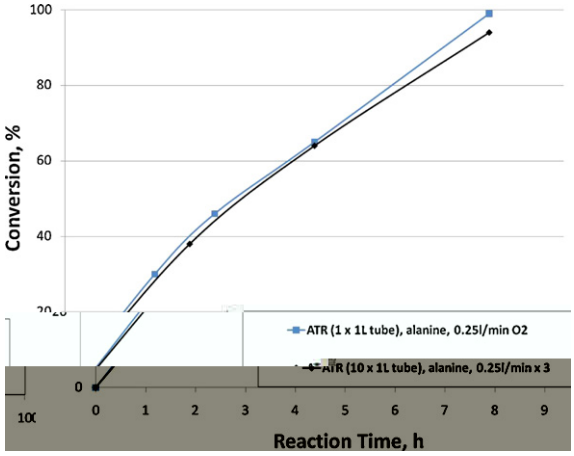

Fig. 15 – Scale-up in the ATR. Enzyme load = 21 g/L, oxygen: 0.25 L/min ATR. In the 1× 1 L case one O_2 input of 0.25 L/min is used but in the case of the 10× 1 L, 3× 0.25 L/min inputs are used.

the biocatalyst under different agitation conditions in each separate reactor system.

The main advantage of scaling up in continuous mode is to avoid the loss in performance seen in Fig. 12 when using bigger reactors. Because of the geometry, the similarity between different sizes and the dynamic mixing technique, it is possible to achieve virtually the same result when scaling up from 1 to 10 L without seeing the losses already evident when going from 1 to 4 L in batch (Fig. 15).

It is anticipated that further improvements to this process can be achieved by multi stage oxygen addition (to optimise the gas/liquid dispersion within the reactor) and higher operating pressures (to increase the oxygen uptake rate).

6. Conclusions

This reaction is mixing sensitive which has significant implications for scale up. Not only does mixing efficiency suffer with scale up but high agitation speeds on large vessels present a variety of problems such as high cooling loads, seal wear, shaft stability and baffling. The commercial implications of scaling up this process under batch conditions would be high capital and operating costs. These results report a method and technology allowing rapid transfer from the research level to process development without the time-consuming optimisation of methods from laboratory scale to production scale.

The advantages of the two flow reactors over a batch system are as follows:

- *Flexible capacity*: 0.01–0.1 L operating volume in ACR, 0.25–10 L in ATR;
- *Flexible heat transfer*: external cooling/heating jacket giving up to 1000 m^2/m^3;
- *Mixing*: strong radial mixing which is independent of residence time;
- *Plug flow*: good plug from minutes to hours;
- *Low pressure drop*: large flow channels result in low pressure drops;
- *Good product handling*: large well mixed channels give good handling of 2/3 phase mixtures;
- *Simple design*: simple tubes with no rotating shafts, seals or baffles;
- *Materials of construction*: choice of stainless steel, alloys or plastic.

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