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Membrane Reactor System for Parallel Continuous Screening and Characterisation of Biocatalysts

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Reliable bioprocess design requires the estimation of "true" kinetic parameters, which should be the same for both scales - the lab-scale and the process scale. However, commercially available screening and characterisation systems are mostly operated in batch/fed-batch mode, which is not sufficient to describe the continuous operational mode that is typically used for native enzyme applications. So, a new concept of membrane based screening and characterisation system that enables physical retention of the dissolved catalyst is proposed and developed.

1. Introduction

Biocatalysis offers versatile advantages over chemical catalysis. Biocatalysts do not only accelerate the reactions millionfold at moderate temperature and pressure, they are usually highly stereo-, regio- and chemoselective. Thus, the use of biocatalysts in industrial applications saves energy costs and makes the product purification a lot easier. The biocatalysis itself can be divided into catalysis with whole cells, with extracted (native) enzymes as well as with immobilised enzymes. The catalysis with native enzymes benefits from high catalyst concentration and specific activity as well as the necessity to immobilise the enzyme on a carrier. However, a few drawbacks of the catalysis with native enzymes are also known: the enzymes have to be recovered in the reactor space and they tend to loose their activity over reaction time, by high shear stress or by pH and temperature fluctuations. Although the challenge of enzyme recovery is currently solved by means of ultrafiltration membranes at technical scale, for a successful process development as well as for an adequate scale-up proper process characterisation is indispensable. At the early stage of process development parallel bioreactor systems are used. Table 1 summarises main properties of a few commercially available and developed stirred tank reactor (STR) systems, which are generally favoured for secondary screening. From table 1 it can be deduced that despite a lot of effort which was made to facilitate the fast transfer of fermentation data from micro to macro scale, the enzyme membrane reactor (EMR) applications are still underrepresented in this scale. Although the presented systems might give a rough idea about the reaction kinetics and process parameters, this data has only limited transferability to a continuous EMR. The exceedingly important data, such as enzyme deactivation, altering of reaction kinetics in continuous operational mode as well as the long-term membrane performance, which are considered as the most critical factors for scale-up of EMR-processes (Wöltinger et al., 2005), stay completely obscured.

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Table 1: Overview of commercially available parallel STR-systems for biocatalysis

System	Volume [mL]	Instrumentation	№ of Reactors	Operational mode
Harms et al. (2006)	2	pH, DO, OD	24	batch
Kusterer et al. (2008)	12	T, pH, DO, OD, flow rate	48	batch
Cellstation/Fluorometrix	35	T, pH, DO, OD	12	batch
Xplorer/Bioxplore	30-100	T, pH, DO, flow rate	8	fed-batch
Explorer/Medicell Oy	100-500	T, pH, DO, OD, flow rate	15	fed-batch
Biostat/Sartorius AG	500-10000	T, pH, DO, flow rate, foam	6-12	fed-batch

Regarding the drawbacks of the existing STR-systems for biocatalysis mentioned above, a new type of membrane-based screening and characterisation system was recently proposed (Lyagin et al., 2010). The desired features of the system are:

- small-scale (< 100 mL)
- continuous operation mode with homogeneously distributed catalysts
- monitoring and control of T, pH, hydraulic retention time (HRT) as well as enzyme activity
- parallel operation (≥ 2)
- long-term operational mode (≥ 100 h)
- precise dosing of small volumes of additional components (co-factors, substrates etc.)
- knowledge of fluid dynamics, power input as well as shear stress in the reactor

Most of these have already been implemented (Lyagin et al., 2010) and shall be further validated here along with the installation of a precise dosing system.

2. Materials and methods

2.1 Implementation of the screening and characterisation system

The main components of the developed screening and characterisation system (Figure 1A) are: membrane reactor (1, made in the departmental workshop of *TU Berlin*, *Germany*), pressure regulator (2, MPPE-3, *Festo AG*, *Germany*), mixing device (3, MIX1, *2MAG*, *Germany*), thermostat (4, *Thermo Haake GmbH*, *Germany*), precision balance (5, ALT 310, *Kern & Sohn GmbH*, *Germany*), pH-sensor (6, QP930X, *ProSense*, *Netherlands*) as well as a safety valve (7, EC-218.12*, Riegler GmbH*, *Germany*).

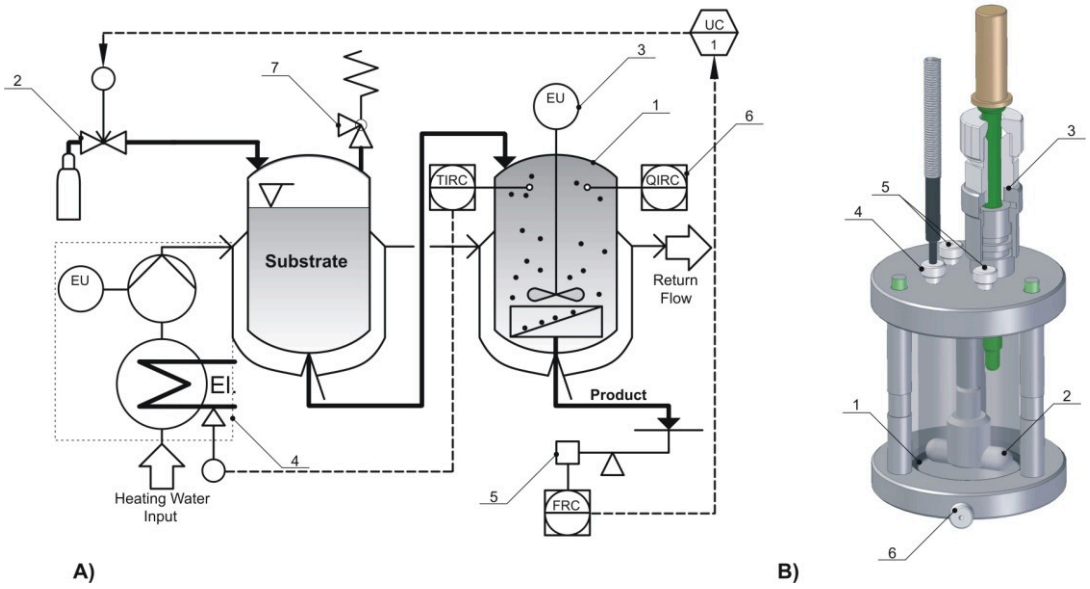

Figure 1: A) Simplified flowsheet of the new screening and characterisation system
B) Design of the membrane reactor

The membrane reactor itself was designed based on a commercially available dead-end test cell from Merck-Millipore AG (XFUF-047) with a working volume of approx. 90 mL and a membrane surface area of 14 cm^2. The main components of the constructed membrane reactor (figure 1B) are: membrane (1), magnetic stirrer (2), connectors for pH and temperature sensors (3 and 4, respectively) as well as connectors for inlet and outlet pipes (5 and 6).

2.2 Model reactions

Two model reactions were chosen for the proof of principle and evaluation of the concept, respectively: hydrolysis of cellulose and hydrolysis of N-acetyl-L-methionine (NAM). The hydrolysis of cellulose is not only an important reaction for the food and energy sectors, it is also strongly inhibited by its products (cellobiose and glucose) and has a high fouling potential, which makes it especially interesting for the screening system's evaluation. This model reaction was used for batch and semi-continuous experiments. The enzymatic hydrolysis of NAM represents a very well-known and industrially important reaction, which is probably the first industrial application of an immobilized enzyme (Tosa et al., 1969). In 1982, Degussa presented a similar process for the production of L-methionine (MET) but with a soluble acylase (Leuchtenberger et al., 1984). This model reaction with a soluble acylase has been used to reproduce a continuous operation mode.

2.3 Chemicals and membranes

For the hydrolysis of cellulose, the cellulase from *Trichoderma reesei* (C8546, *Sigma-Aldrich Corp.*, *USA*) was used. As substrate for this reaction the α-cellulose (C8002, *Sigma-Aldrich Corp*., *USA*) was used. This reaction was buffered by means of sodium acetate buffer. For the hydrolysis of NAM (22003320, *Molekula GmbH*, *Germany*), the acylase I from *Aspergilus melleus* (534862, *Sigma-Aldrich Corp*., *USA*) as well as tris buffer were used. Polyethersulfon (UP005, 5 kDa and UP010, 10 kDa, *Microdyn Nadir GmbH*, *Germany*) and regenerated cellulose (UC010, 10 kDa, *Microdyn Nadir GmbH*, *Germany* and Hydrosart 14429, 5 kDa, *Sartorius AG*, *Germany*) membranes were used.

2.4 Enzyme activity tests and analytical methods

Enzyme activity test for cellulase was performed in accordance with Sigma-Aldrich control test procedure (1995), where 1 unit of cellulase liberates 1 µmol/h of glucose from Sigmacell®-cellulose (S3504, *Sigma-Aldrich Corp*., *USA*). Enzyme activity for acylase I was measured at T = 37 °C, pH = 8.0 and $C_{NAM,0}$ = 20 mM, where 1 unit of acylase I liberates 1 µmol/h of MET from NAM.

2.5 Analytical methods

The total concentration of produced sugars was measured using refractometry (DD-7 precision refractometer*, ATAGO Co*, *Ltd*., *Japan*). The concentration of the produced MET was measured by means of spectrometry (Specord 200, *Analytic Jena AG*, *Germany*).

3. Results and discussion

3.1 Flux control

The maintenance of a desired hydraulic retention time (HRT) is of high importance at technical scale and shall thus be achievable also with the developed screening system.

When components with high fouling potential are present, it is not anymore sufficient to maintain the actuating variable (in our case the feed pressure) at a desired level but a closed-loop control should be integrated. Figure 2 presents results from the developed flux control concept and shows the typical feed pressure development in the membrane reactor over filtration time, if hydrolysis of cellulose is carried out. As can be seen, the integrated controller should not only be able to compensate the permeability decline but also be stable even by high flux fluctuations. Figure 2 also proves the stability and accuracy of the integrated flux control system: the desired flux was maintained during the whole operation (over 50 h) with a standard deviation of ±2.5 %. The PID-controller settings were determined as described previously (Lyagin et al., 2010).

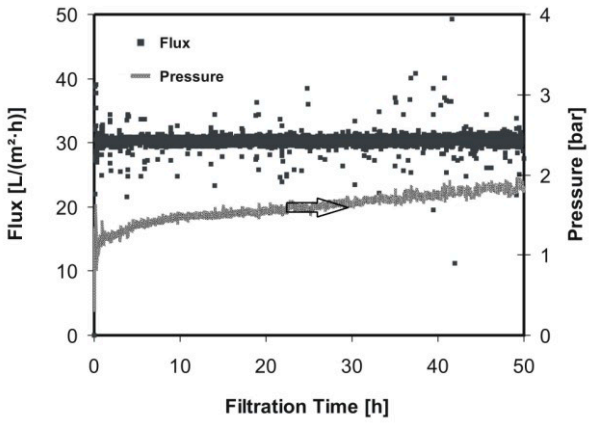

Figure 2: Flux control over time, $C_{cellulose,0}$ = 25 g/L, $C_{Enzyme,0}$ = 560 U/L, T = 40 °C, pH = 4.66, UP010

3.2 Batch, semi-continuous and continuous fermentations

The cellulose hydrolysis was carried out in batch and semi-continuous operation modes. In case of semi-continuous operation mode, no substrate was added continuously to the reactor room. However, the product was continuously removed from the reactor space to prevent the product inhibition (by means of permeate removal). The initial added insoluble substrate as well as the enzymes were thereby retained in the reactor space; the loss of permeate itself was compensated by addition of acetate buffer instead of substrate. The hydrolysis of NAM was carried out in continuous operation mode, where the fresh substrate solution was added all the time. The conversion degrees were calculated under the assumption of an ideally mixed reactor. In case of batch and continuous operation modes those were calculated as:

$$Conversion_{Batch/Conti}(t) = \frac{C_{Sample}(t)}{C_{S,0}} \cdot 100\% \quad (1)$$

For the semi-continuous operation mode the conversion degree was calculated as:

$$Conversion_{Semi-Conti}(t) = \frac{V_{Collected}(t) \cdot \overline{C}(t) + (V_{Sample} + V_{Reactor}) \cdot C_{Sample}(t)}{V_{Reactor} \cdot C_{S,0}} \cdot 100\% \quad (2)$$

Figure 3 shows the comparison of cellulose conversions in batch and semi-continuous operation modes, under otherwise equivalent conditions. The error bars represent measurements from two EMRs, which were integrated in the system in parallel (acc. to Figure 1). As can be seen, a good reactor parallelism was achieved (less as ±4.5 % deviations from the averaged values (Lyagin et al., 2010)). Furthermore, the repetitive fermentations show a good reproducibility of less than ±4 % deviations on average (data not shown). The comparison with batch fermentations from literature which are done under equivalent conditions shows less than 15 % deviations on average, which is reasonably good, considering the fact that the enzyme concentration could be compared only in terms of mg/L. The fermentations itself show, as expected, the product inhibition and as a result a conversion increase with a decrease of HRT. Compared with the batch operation, the semi-continuous fermentation with HRT of 3 h produces nearly 60 % more product, which clearly indicates the potential of EMRs for this application.

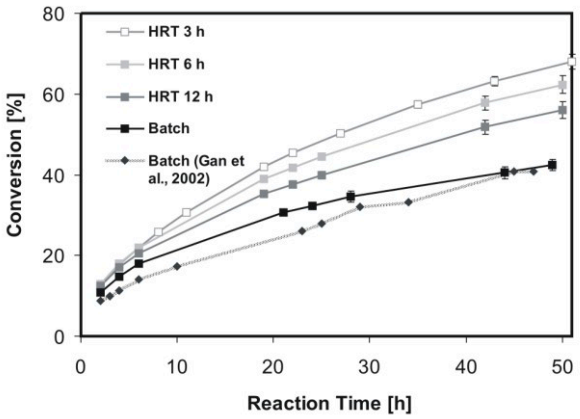

Figure 3: Cellulose conversion during batch and semi-continuous operations with HRTs from 3-12 h, $C_{cellulose,0}$ = 25 g/L, $C_{Enzyme,0}$ = 1120 U/L (equivalent to 200 mg/L by Gan et al., 2002), T = 40 °C, pH = 4.66, UP010, adapted from Lyagin et al. (2011)

Figure 4 shows the comparison between two continuous NAM-fermentations, with error bars as expected analytical errors. Both fermentations were carried out under the same conditions; however, the used membranes were different. The Hydrosart membrane has a MWCO of 5 kDa and was observed to reject approximately 99.9 % of acylase I, whereas the UC010 with a MWCO of 10 kDa rejected 99.5 - 99.9 % (data not shown). From figure 4, it is clear that the conversion decline cannot be caused by enzyme leaching, since even by considering a minimal enzyme rejection of UC010 from 99.5 %, about 90 % of initial enzymes should be available after 140 h of operation. That would cause a conversion decline of approx. 3 %, the observed decline however is about 9 %. One of the reasonable explanations of this conversion decline is the different membrane materials. Indeed, the Hydrosart membrane is reported to be an extremely low protein binding membrane (Sartorius, 2010), whereas the UC010-membrane is a typical regenerated-cellulose membrane not optimised for enzyme applications.

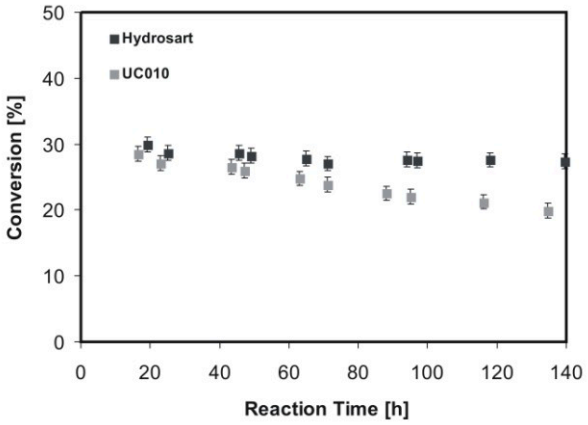

Figure 4: Hydrolysis of NAM during continuous operation with two different membranes at HRT = 6 h, $C_{NAM,0}$ = 20 mM/L, $C_{Enzyme,0}$ = 3600 U/L, T=30 °C, pH=8.0

4. Concluding remarks

The potential of the developed screening and characterisation system was shown on the basis of two industrially important enzymatic reactions. Despite the high fouling potential of the cellulose hydrolysis, the HRT could be controlled over more than 100 h with a precision of ±1 %. The parallelism and reproducibility of the fermentations were also demonstrated. Furthermore, the used semi-continuous operation mode gave nearly 60 % more product compared to the batch fermentation. In a second step, the hydrolysis of NAM was carried out in continuous operation mode. Thereby it was possible to clearly distinguish between two different types of enzyme activity decreases, caused by enzyme depletion and enzyme adsorption. So, in order to describe the reduction of the enzyme activity as well as to control it, a concept for precise fluid dosing was worked out and realised. The dosing of small amounts of liquids from 0.25-10 mL with ±3.5 % precision is possible. The system is further modified to include automatic enzyme activity and pH control.

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