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ScienceDirect
Journal of Biotechnology 132 (2007) 202–208
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

# Integrated processing and multiple re-use of immobilised lipase by magnetic separation technology

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Received 3 January 2007; received in revised form 2 May 2007; accepted 25 May 2007

**Abstract**

High-gradient magnetic separation based processing is demonstrated for the semi-continuous multicycle re-use of a lipase immobilised on magnetic microparticles. The lipase of *Candida antarctica* A-type (CALA) was immobilised on polyvinyl alcohol coated magnetic particles (1–2 μm diameter) with epoxy functionalisation. The immobilised CALA was used to hydrolyse a model oil-water 2-phase-system composed of a phosphate buffer with tributyrin at up to 3 l scale. The immobilised enzyme was subsequently recovered in a magnetic filter using high-gradient magnetic separation and reapplied in repeated cycles of hydrolysis and recovery. Two different temperatures of 30 and 50 °C, tributyrin concentrations (0.12 and 35 g $l^{-1}$) and reaction times were tested. In each case the reaction was followed by pH titration using NaOH, as well as by HPLC analysis. Consecutive cycles were conducted for each reaction condition and in total the immobilised CALA was subjected to 20 recovery and re-use cycles, after which ~14% of the initial specific activity still remained.


*Keywords:* Enzyme; Immobilisation; *Candida antarctica* lipase type-A; CALA; 2-Phase-system; HGMS; Recovery

## 1. Introduction

Enzyme-catalysed chemical transformations are now widely recognised as practical alternatives to traditional (non-biological) organic synthesis, and as efficient solutions to synthetic processes (Koeller and Wong, 2001). Lipases are amongst the most important biocatalysts carrying out novel reactions in aqueous and non-aqueous media. This is primarily due to their ability to utilise a wide spectrum of substrates, to have high stability towards extremes of temperature, pH and organic solvents and to show chiral, regio- and enantioselectivity (Koeller and Wong, 2001). In addition, there is great potential for using lipases to produce alkyl esters by direct conversion of triacylglycerols, which can subsequently be used to synthesise value-added products, for instance biodiesel or biodegradable lubricants (ester oils) (Brenneis et al., 2004). One option to render such processes more economical is the use of immobilised biocatalysts instead of free enzymes, which cannot be recovered and re-used.

Recent work on magnetic separation of immobilised enzymes has been published in which the recovery and multiple re-use of an immobilised lipase was investigated in bench scale (Guo and Sun, 2004). Lipase from *Candida rugosa* was immobilised on hydrophobic and superparamagnetic microspheres and used as catalyst for esterification reactions. The enzyme was recovered by a bar magnet and exhibited good reusability in repeated batch reactions of 2 ml. In the solvent free system used, 73% of the initial activity was found after 10 cycles (Guo and Sun, 2004). In another study a glucose oxidase-magnetite nanoparticle bioconjugate was used for glucose sensing (Rossi et al., 2004). The glucose oxidase-coated magnetic particles remained active for two successive runs and thereafter activity loss was seen. After five recycles, 50% of the activity remained. The authors speculated that the substantial loss of activity was probably due to loss of magnetic particles during the magnetic separation and re-dispersion cycles rather than a decrease in specific activity of the immobilised enzyme (Rossi et al., 2004).

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0168-1656/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2007.05.029

Hoffmann et al. (2002) and Meyer et al. (2005) developed a fully automated high-gradient magnetic separation system (HGMS) for the isolation of selected proteins from whey, which could potentially be adapted to the separation and re-use of immobilised enzymes. The HGMS system contained a magnetic filter cassette (~46 ml volume) into which loaded magnetic particles were pumped and captured. Non-magnetic substances and the reaction mixture flowed through the filter. The magnetic particles could then be washed or eluted and flushed out of the cassette by any buffer or liquid chosen, in a process termed high-gradient magnetic fishing (HGMF) (Hubbuch et al., 2001; Meyer et al., 2005). In the current work, adaptation of HGMS/HGMF was investigated for a mini-pilot scale semi-continuous multi-cycle process for the re-use of *Candida antarctica* A-type lipase (CALA) immobilised on superparamagnetic microparticles.

## 2. Material and methods

### 2.1. Magnetic particles

Poly vinyl alcohol epoxy-activated magnetic beads (M-PVA E02) were obtained from Chemagen Biopolymer Technologie AG (Baesweiler, Germany) and according to the manufacturer they contained 50–60% magnetic material. Furthermore, the density of functional groups as determined by the manufacturer was 200 μmol epoxide $g^{-1}$ particles and they were stored in acetone at 4 °C. The epoxy terminated M-PVA particles used did not need additional activation prior to immobilisation of the lipase.

### 2.2. Candida Antarctica lipase

A-type lipase (CALA), Novozym L868 was purchased from Novozymes A/S (Bagsværd, Denmark). The stock solution had a protein concentration of 10 mg $ml^{-1}$ as determined with the Bradford assay and an activity of 1100 $U_{p\text{-NPP}}$ $ml^{-1}$ was found, where one unit ($U_{p\text{-NPP}}$) is defined as the cleavage of *p*-nitrophenol palmitate (*p*-NPP) at a rate of 1 μmol $min^{-1}$. The resulting specific activity was thus measured to be 110 $U_{p\text{-NPP}}$ $mg^{-1}$ lipase. *p*-NPP, and tributyrin were obtained from Sigma–Aldrich (Steinheim, Germany) and all other chemicals were of analytical grade.

#### 2.2.1. Immobilisation of the lipase

CALA was immobilised on M-PVA E02 particles as described in Schultz et al. (2007). In brief, M-PVA E02 particles were resuspended to give a concentration of 30 mg $ml^{-1}$ in 0.1 M sodium phosphate buffer (pH 7). The lipase was then added, giving an enzyme concentration of 0.2 mg $ml^{-1}$ and incubated with shaking overnight at room temperature. Immobilisation was stopped by magnetic capture of the particles and washing as described in Schultz et al. (2007). The supernatant after immobilisation, as well as the washing fractions, were collected and analysed for specific activity and protein content. Two batches of 30 mg of immobilised enzyme were made exactly as described above and had an activity of 600 $U_{p\text{-NPP}}$ $g^{-1}$ particle (batch 1) and 433 $U_{p\text{-NPP}}$ $g^{-1}$ particle (batch 2) and were used in the bench scale and stability studies, respectively. Subsequently the procedure was scaled up linearly to produce 5 × 600 mg batches which were combined (batch 3) giving an average activity of 420 ± 29 $U_{p\text{-NPP}}$ $g^{-1}$ particle and were used for HGMS studies.

### 2.3. Study of enzyme stability

The free and immobilised CALA (batch 2) were resuspended in 0.1 M sodium phosphate buffer at different pH values (pH 6, 7, 8) and then incubated at different temperatures (4 and 25 °C). Samples were taken over a 38 day period and the specific activity of free and immobilised CALA was determined using a spectrophotometric assay with *p*-NPP as substrate.

### 2.4. Bench scale re-use studies with immobilised CALA

Small-scale studies were conducted in a temperature controlled and stirred batch reactor with a final volume of 30 ml. The model reaction solution contained 29 ml of 10 mM sodium phosphate buffer (pH 8) and 1 ml tributyrin and was kept homogenised with an overhead stirrer (Metrohm SM702) at medium speed. The reaction was started by introducing 20 mg of the immobilised enzyme (batch 1) with a bead related specific activity of 600 $U_{p\text{-NPP}}$ $g^{-1}$ bead. The reaction was followed by pH titration with 50 mM (NaOH) to hold the pH constant at 8.0 and the initial slope was used to calculate the reaction rate. After 0.83 h the immobilised enzyme was collected with a bar magnet (0.3 T) placed on the wall of the vessel and the reaction contents were collected for analysis. The immobilised enzyme was then washed one to three times with 10 mM phosphate buffer (pH 8) and centrifuged for 90 s at 10,000 × *g* in a bench top microfuge. The above sequence of events comprised one cycle and subsequently the washed immobilised enzyme was then added to fresh reaction solution for a further cycle. Particle loss after each cycle was determined gravimetrically.

### 2.5. Recovery and multiple re-use of immobilised CALA by HGMS

Mini-pilot scale studies were conducted in a temperature controlled (30 or 50 °C) and stirred batch reactor with a final volume of 300 or 3000 ml. The model reaction solution contained 10 mM sodium phosphate buffer (pH 8) and 0.345 or 10 ml of tributyrin and was kept homogenised with an overhead stirrer at medium speed. The reaction was then started by introducing up to 3 g of the immobilised lipase (batch 3) with a bead related specific activity of 420 $U_{p\text{-NPP}}$ $g^{-1}$. The reaction was followed by pH titration with 50 mM NaOH, which kept the pH constant at 8, and the initial slope was used to calculate the reaction rate. Samples were taken for HPLC analysis and immediately frozen at −80 °C to avoid further hydrolysis of unreacted tributyrin to glycerol and butyrate. After the reaction had proceeded for the desired time, the suspension was processed using the HGMS apparatus, the immobilised enzyme washed once with 10 mM phosphate buffer (pH 8), recovered and used for the next cycle. Particle loss after each cycle was determined gravimetrically. Four separate multicycle use and re-use campaigns were con-