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Micro-scale process development of transaminase catalysed reactions†‡

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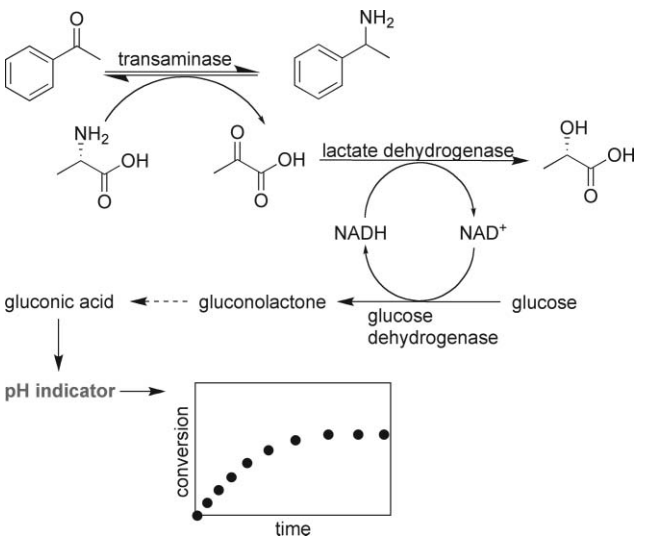
A micro-scale technique has been developed for the process development of transaminase catalysed reactions. This pH indicator based, colorimetric assay can be used to investigate and optimise reaction conditions at 100 μL scale. Enzyme activity and stability as a function of various reaction parameters, including temperature, pH and co-solvent concentration, have been determined. Additionally, reactions have been scaled up from 100 μL to 25 mL under the optimal reaction conditions identified by the micro-scale process development activities. Excellent conversion (>99%) and enantioselectivity (>99% ee) were obtained.

Introduction

In view of the increasing demand for enantiomerically pure chiral amines as building blocks for the development of biologically active compounds including pharmaceuticals and agrochemicals,[1] attention is turning to the use of transaminase enzymes which offer a potentially efficient and scalable option. Transaminases catalyse the transfer of an equivalent of ammonia from an amine donor, to a prochiral ketone substrate, thereby creating the C–N bond as well as establishing the key stereogenic centre in a single step.[2] Attention recently has focused on a number of issues relating to the use of transaminases including, (i) methods for shifting the equilibrium towards product formation,[3] (ii) sources of amine donor,[4] (iii) cloning and over-expression of new transaminases,[5] (iv) the development of multi-enzyme systems for the deracemisation of racemic amines,[6] (v) kinetic resolution of racemic amines[7] and (vi) approaches to the scale-up of transaminase-catalysed processes.[8] Although the number of commercially available transaminases has increased over the past few years, the timeline for evaluating biocatalysts has traditionally lagged behind chemo-catalysis,[9] and process development remains a key bottleneck in biocatalyst evaluation. In order to address this issue, we herein report a pH indicator based micro-scale process development technique for the optimisation of transaminase catalysed reactions. Micro-scale techniques are emerging as useful methods for the identification of critical process parameters in various areas including: process optimization, bioavailability studies, formulation shelf life analysis, and microbial fermentations.[10–12] The combination of shorter process development timelines and reduced material requirements for the often expensive synthetic substrates, makes micro-scale process optimization techniques a promising technology for wider application. Although pH indicator based assays have been extensively used in hydrolase chemistry,[13–16] this is the first report of a pH indicator based development technique for transaminases.

Results and discussion

Scheme 1 shows the pH indicator based micro-scale assay. The transamination of acetophenone to methylbenzylamine served as the desired transformation to be optimized. The ketone is transaminated using alanine as the amine donor. The pyruvate byproduct is reduced to lactate using lactate dehydrogenase. The removal of the keto-acid byproduct serves to both eliminate transaminase inhibition by pyruvate and drive the reaction equilibrium.[17] Lactate dehydrogenase requires the cofactor NADH, which is recycled using glucose dehydrogenase and glucose. The gluconolactone product from the cofactor recycle reaction then spontaneously hydrolyzes at alkaline pH to gluconic acid, causing a pH change. Conversion is determined based on the absorbance change of a pH indicator dye present in the reaction solution.



Scheme 1 pH indicator based micro-scale transamination assay for monitoring conversion.

Three pH indicator dyes were evaluated for use in the micro-scale assay. Bromothymol blue (BTB), phenol red and cresol red were chosen for their useful pH range between pH 6 and pH 9. Fig. 1 shows the absorbance change of each indicator dye as a function of solution pH. Phenol red was chosen as the optimal pH indicator dye due to its steep linear absorbance curve in the desired pH range.

The pH response of the assay can be tuned by adjusting the buffer strength of the reaction solution. Fig. 2 shows the

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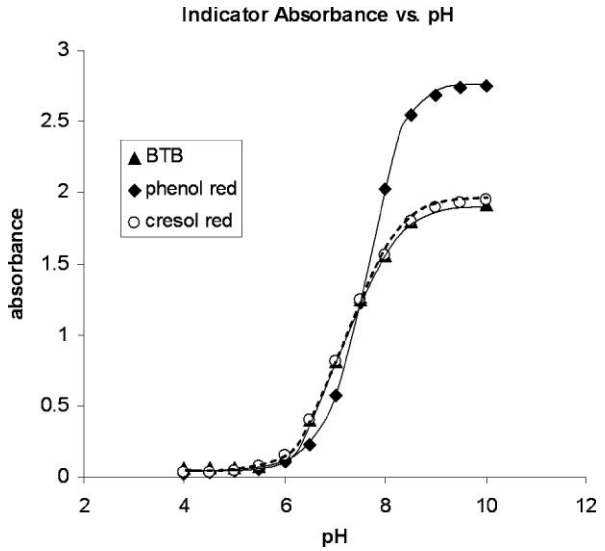

Fig. 1 pH indicator absorbance *vs.* pH profile for bromothymol blue (BTB), phenol red and cresol red. The absorbance for BTB, phenol red and cresol red was monitored at 620 nm, 560 nm and 570 nm respectively.

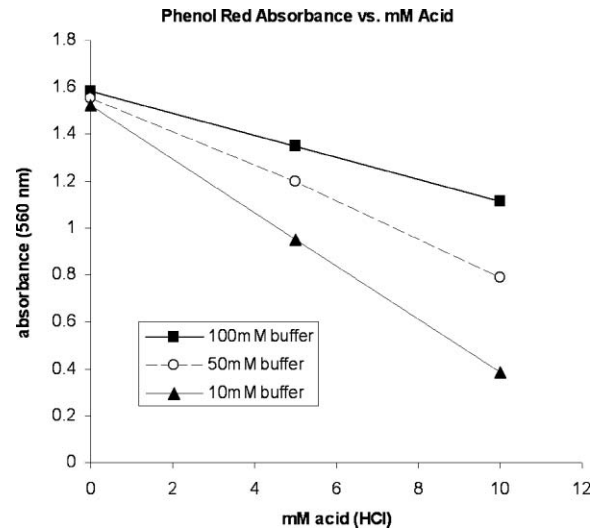

Fig. 2 Phenol red absorbance *vs.* mM acid (HCl) added to solution. The absorbance was measured at a wavelength of 560 nm.

absorbance change *versus* concentration (mM) of acid addition of reaction solutions containing the phenol red indicator and different concentrations of potassium phosphate buffer.

A comparison between the pH indicator based micro-scale assay and a conventional HPLC based assay was conducted to determine the accuracy of the micro-scale measurements of conversion. Reactions were run under identical conditions for 24 h with 10 mM acetophenone substrate in 50 mM potassium phosphate buffer. 50 mM buffer was used because it allowed for accurate determination of conversion, while also keeping the pH of the reaction within 0.1 pH units of the starting pH. One reaction contained the phenol red pH indicator dye and was run at 100 µL scale in a plate spectrometer. The absorbance data obtained was converted to conversion data using the standard curve generated in Fig. 2. The other reaction was run at 1 mL scale and analyzed for conversion by HPLC. Fig. 3 shows that both analytical methods produced comparable data over the entire reaction progress.

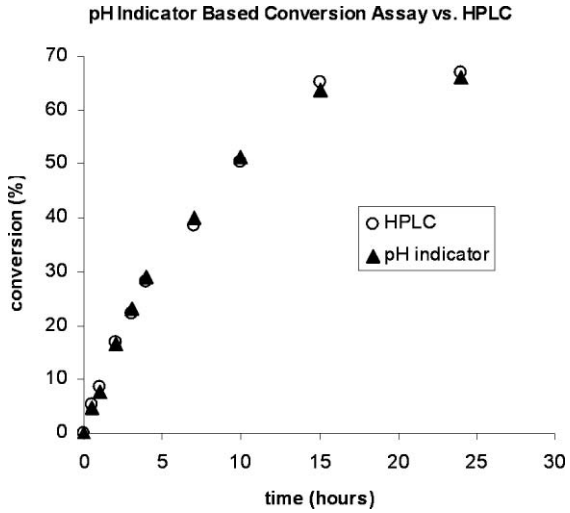

Fig. 3 Comparison of pH indicator based conversion assay to standard HPLC based assay. Reactions were run with 10 mM acetophenone substrate in 50 mM potassium phosphate buffer at 20 °C.

Process development was then carried out using the micro-scale assay to optimize reaction conditions. The rate of reaction was determined at various pH values between pH 7 and pH 8.5 (Fig. 4). pH 8 exhibited the highest reaction rate in the transamination system.

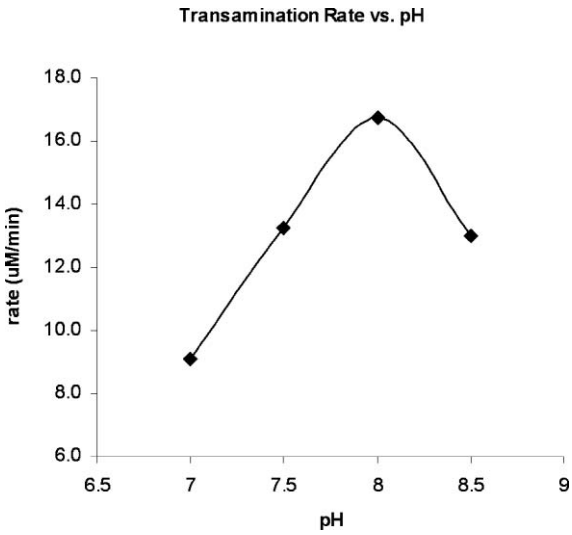

Fig. 4 Transamination rate *vs.* reaction pH. Reactions were run at 20 °C.

Enzyme activity and stability as a function of temperature were then evaluated to determine the optimum reaction temperature for this multi-enzyme system. Fig. 5 shows the reaction rate *vs.* temperature determined using both the micro-scale assay and conventional HPLC assay. The data show excellent correlation. Enzyme stability *vs.* temperature was also determined and is shown as the enzyme half-life. The optimal temperature for this transamination reaction was determined to be 30 °C, as a distinct decrease in enzyme stability was observed at >30 °C.

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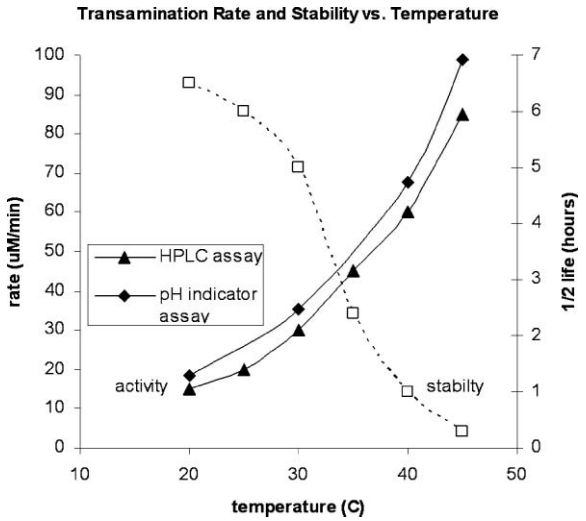

Fig. 5 Transamination rate and enzyme stability *vs.* reaction temperature. Reaction rate *vs.* temperature was determined using both the micro-scale assay and conventional HPLC assay.

Various organic co-solvents were evaluated for their effect on reaction rate and enzyme stability. Fig. 6 shows that only the addition of MeOH exhibited a higher reaction rate compared to that of buffer alone with no organic co-solvent. However, enzyme stability suffered with all of the co-solvents tested.

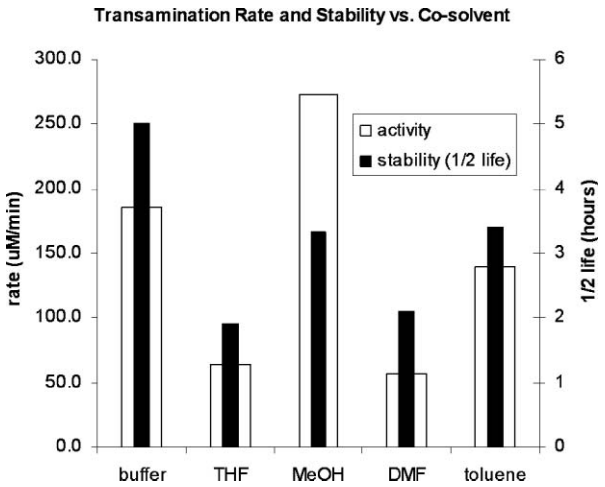

Fig. 6 Transamination rate and stability *vs.* organic co-solvent addition. 10% v/v organic co-solvent was used.

The micro-scale process development activities determined the optimal reaction conditions to be: 30 °C, pH 8.0, with an all buffer (no organic co-solvent) reaction system. The optimal reaction conditions then were scaled up from 100 µL to 25 mL scale. The larger scale transamination reaction was run with pH control using automated base titration to keep the reaction at its optimal pH. Fig. 7 shows that >99% conversion was achieved in 9 h. The enantiomeric excess of the *R*-methylbenzylamine product, determined by normal phase HPLC, was >99% ee.

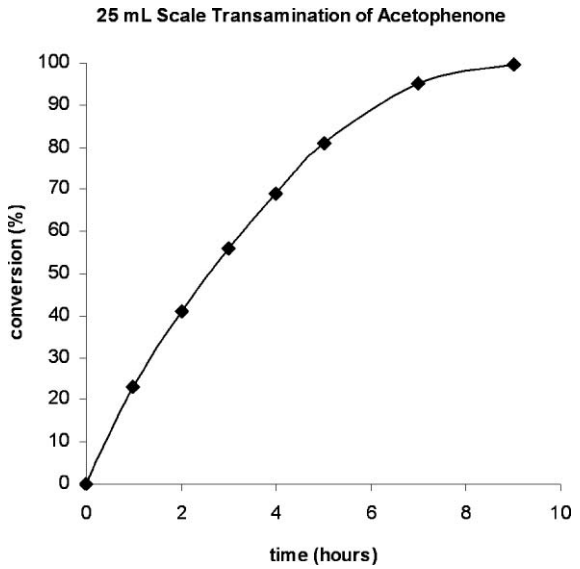

Fig. 7 25 mL scale transamination of acetophenone using optimised reaction conditions (pH 8.0, 30 °C). Reactions were run with pH control using automatic titration of base (2 M NaOH).

Conclusions

In summary, we have developed a convenient and rapid micro-scale process development strategy for transamination reactions. Tuning the buffer strength of the pH indicator based assay system allows tracking of the complete conversion profile, enabling the determination of not only initial rates of reaction, but also enzyme stability over time. The optimization of various process parameters including reaction pH and temperature, and evaluation of organic co-solvents was followed by scale-up to 25 mL scale. This system is also ideal because a direct scale-up from micro-scale to process scale can be made with no further modification once suitable reaction conditions are identified. This ability for direct scale-up from 100 µL scale to first material delivery scale greatly reduces the process development timeline.

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