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Development of a high throughput screening tool for biotransformations utilising a thermophilic L-aminoacylase enzyme

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ABSTRACT

Micro-reactors containing a monolith-immobilised thermophilic L-aminoacylase, from *Thermococcus litoralis*, have been developed for use in biotransformation reactions and a study has been carried out to investigate the stereospecificity and stability of the immobilised enzyme. The potential to use the developed micro-reactors as a tool for rapid screening of enzyme specificity was demonstrated, confirming that the L-aminoacylase showed a similar substrate specificity to that previously reported of the free enzyme. From this baseline, the technique was employed as a tool to evaluate potential unreported substrates with *N*-benzoyl- (L-threonine, L-leucine and L-arginine) and *N*-acetyl- (D,L-serine, D,L-leucine, L-tyrosine and L-lysine) protecting groups. The order of preferred substrates was found to be Phe > Thr > Leu > Arg for *N*-benzoyl substrates and Phe ≫ Ser > Leu > Met > Tyr > Trp for *N*-acetyl substrates.

It was found that by using the micro-reactor a significantly smaller quantity of enzyme and substrates was required. It was shown that the micro-reactors were still operational in the presence of selected organic solvents, such as ethanol, methanol, acetone, dimethylformamide (DMF) and dimethylsulfoxide (DMSO). The results indicated that a combination of a small amount of an appropriate solvent (5% DMSO) and a higher reaction temperature could be employed in biotransformations where substrate solubility was an issue.

1. Introduction

Enzymes are continuing to be exploited for industrial purposes [1,2] and in particular, this area has received attention from pharmaceutical companies for the production of high value chemicals and new drug intermediates [3]. The use of enzyme catalysts allows high synthetic performance under mild conditions, minimising problems such as isomerisation, racemisation and epimerisation; which can reduce the quantity of side products that are generated during the process. In addition, enzyme reactions are stereoselective and therefore can produce optically pure products. The number of new pharmaceutical compounds on the market that are chiral is expected to increase to 70% by 2010 [4].

Enzymes are however often unstable under the necessary operating conditions of the commercial process and enzymatic processes can be more expensive to develop than those using traditional synthetic chemistry. Enzymes isolated from thermophilic organisms have evolved to operate optimally at high temperatures and are inherently more stable to elevated temperatures and organic solvents than their mesophilic counterparts. Although the thermophilic enzymes have optimal activity at elevated temperatures they are also active at lower temperatures which allows a broad operating window.

The L-aminoacylases are important enzymes for industrial applications since they can be used to resolve a racemic mixture of *N*-acyl amino acids to produce a range of L-amino acids and amino acid analogues. We have chosen to use a thermostable L-aminoacylase from the thermophilic archaeon *Thermococcus litoralis* which we have previously cloned, sequenced and overexpressed in *Escherichia coli* [5]. The purified enzyme has been characterised and found to have optimal activity at 85 °C in Tris–HCl buffer at pH 8.0 [5]. It has been shown to exhibit most specificity towards substrates containing *N*-benzoyl or *N*-chloroacetyl protected amino acids (Scheme 1). This is unlike the more common commercially available 'Amano' aminoacylase which has preferential selectivity for acetyl > chloroacetyl ≫ Boc > benzoyl. It is also has different substrate specificity to other thermophilic L-aminoacylases from *Pyrococcus furiosus* [6] and the related *Pyrococcus horikoshii* [7]. The *Thermococcus* L-aminoacylase is a homotetramer of 43 kDa monomers, and has an 82% sequence identity to the aminoacylase from *P. horikoshii* and 45% sequence identity to a carboxypeptidase from *Sulfolobus solfataricus*. Aminoacylase inhibitors, such as mono-*tert*-butyl malonate,

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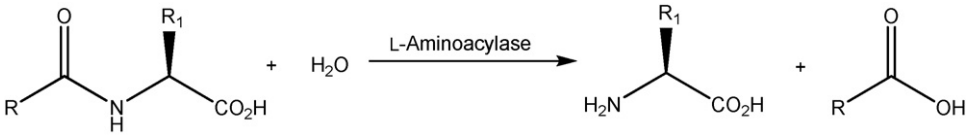


Scheme 1. Reaction catalysed by L-aminoacylase; R represents the *N*-protecting group [2,3].

have only a slight effect on the activity of the *Thermococcus* enzyme [5].

Previous work has been successful in immobilising the *T. litoralis* enzyme [8]. A column bioreactor containing the recombinant L-aminoacylase immobilised onto Sepharose beads was constructed with the substrate, *N*-acetyl-DL-Trp, continuously flowing at 60 °C for 10 days. Using a flow system has the added advantage that any problems with substrate and product inhibition are eliminated.

Microfluidic systems were first developed for analytical chemistry where they are commonly referred to as micro-total analytical systems (μTAS) [9]. More recently micro-reactors, having reaction channels between 200 and 1000 μm, have been used for fine chemical synthesis. It is now well established that chemical reactions conducted in such devices generate products in higher yield, purity and selectivity when compared to batch reactors [10,11]. The advantages of micro-reactors for enzymatic reactions have been reviewed by Miyazaki and Maeda [12].

The use of micro-reactors containing an immobilised enzyme offers an opportunity to minimise the quantities of enzyme, substrate and other reagents required, therefore reducing the costs when compared to a batch reaction. Several approaches have been taken towards immobilising enzymes in micro-reactors; the initial approach tended to be to immobilise the enzyme onto silica or polymer microparticles which were then packed within the micro-reactor [13]. A recent development by us has been to pack the reaction channel with an immobilised gamma lactamase catalyst and to produce an efficient micro-reactor [14]. The immobilisation of the enzyme onto the micro-reactor wall, can overcome problems associated with high back pressures, but the enzyme loading is lower [15]. The use of membranes within channels to immobilise enzymes has also been investigated, however this is difficult to achieve [16].

In comparison to these techniques, monoliths are well-ordered macroporous materials with low flow resistance, high reaction efficiency and good flow-through properties. They can be formed by *in situ* polymerisation making them ideal for use as components in micro-reaction systems. The application of monoliths in enzyme immobilisation in micro-reactors has proved to be successful for glucose oxidase [17] and a protease [18], where the monolith was prepared from a mixture of tetramethoxysilane and methyltrimethoxysilane.

The only reported use of an industrially important acylase enzyme within a micro-reaction system was for an analytical application in which the optical resolution of racemic amino acids was performed [19]. The work reported focused mainly on the extraction of the product L-amino acids from uncleaved D,L-substrates using an in-line aqueous extraction. The enzymatic reaction was carried out on acetyl-D,L-amino acids at 40 °C and using a flow rate of 0.5 μl min^{-1}. Although employing cross-linked polymerised aminoacylase (Amano) with a polylysine matrix on the surface of the micro-channels prevented high back pressure, a reduced surface to volume ratio results compared to immobilisation onto a monolith.

Recent developments involving monolith production over the past decade suggests that by careful selection of monomer and porogenic mixtures a higher degree of functionalisation and minimal back pressures can be achieved [20,21]. Based on this, the present study aimed to develop a micro-reaction system, where the thermophilic L-aminoacylase from *T. litoralis* was immobilised onto polymer monoliths formed inside micro-channels, which has allowed screening of the immobilised enzyme for activity towards a range of potential substrates with different protecting groups (benzoyl-, chloroacetyl-, acetyl-, Cbz- and Boc-) and a variety of amino acids (Phe, Met, Thr, Tyr, Trp, Leu, Ser and Arg), under a range of conditions.

2. Materials and methods

Chemicals were purchased from the sources indicated and were used as supplied; ethylene dimethacrylate, EDMA (98%, Sigma–Aldrich), glycidylmethacrylate, GMA (97%, Sigma–Aldrich), 2,2-dimethoxy-2-phenyl-acetophenone, 3-(trimethoxysilyl)propyl methacrylate (98%, Sigma–Aldrich), DMPA (99%, Sigma–Aldrich), tris(hydroxymethyl) aminomethane, Tris–buffer (Sigma–Aldrich), dodecanol (99%, Sigma–Aldrich), cyclohexanol (99%, Sigma–Aldrich), *N*-benzoyl-L-phenylalanine (Novabiochem), L-phenylalanine (99%, Sigma–Aldrich), *N*-acetyl-L-phenylalanine (98%, Sigma–Aldrich), *N*-chloroacetyl-L-phenylalanine (98%, Sigma–Aldrich), *N*-acetyl-L-tryptophan (98%, Sigma–Aldrich), *N*-benzoyl-L-leucine (98%, Sigma–Aldrich), *N*-benzoyl-L-threonine (98%, Sigma–Aldrich), *N*-acetyl-L-tyrosine (98%, Sigma–Aldrich), *N*-benzoyl-D,L-phenylalanine (98%, Sigma–Aldrich), *N*-*t*-Boc-L-phenylalanine (Sigma–Aldrich), *N*-CBZ-L-phenylalanine (99%, Sigma–Aldrich), *N*-benzoyl-L-arginine (99%, Alfa Aesar), *N*-acetyl-L-methionine (99%, Sigma–Aldrich), *N*-acetyl-L-serine (99%, Sigma–Aldrich), *N*-acetyl-D,L-leucine (98%, Sigma–Aldrich), *N*-acetyl-L-lysine (98%, Sigma–Aldrich). Ethanol and acetonitrile were HPLC grade and purchased from Fisher Scientific.

2.1. Preparation of monolithic micro-channels

The borosilicate glass micro-reactor used in this study was prepared 'in house' using standard fabrication techniques [22] and had channel dimensions of 100 μm depth, 300 μm width and 15 mm length. Poly(glycidylmethacrylate-*co*-ethylenedimethacrylate) monoliths were formed inside the micro-reactor channels using photoinitiation (Fig. 1) [23]. Fig. 1a shows where the monoliths were formed within the micro-channels and Fig. 1(b) is a schematic illustrating how the system was utilised for biotransformation reactions. The channel surfaces of the micro-reactor were pre-treated to enable covalent attachment of the monolith to the channel walls. This was achieved by firstly treating the channels with 0.2 M sodium hydroxide for 1 h, washing them with water and then filling them with 0.2 M hydrochloric acid for 1 h followed by a water and ethanol wash. The channels were subsequently filled with the silanising agent, 3-(trimethoxysilyl)propyl methacrylate (20% in ethanol, adjusted to pH 5 with acetic acid) and left for 1 h. After this the channels were dried with a stream of nitrogen.

To prepare the monolith, two portions of the monomer solution consisting of GMA and EDMA (3:1) (structures of both monomers are provided in Fig. 1) were mixed with one portion of the porogenic mixture containing 4:1 cyclohexanol:dodecanol, into which 1 wt% of the initiator (DMPA), with respect to total weight of monomers, was dissolved. The mixture was then sonicated to ensure thorough

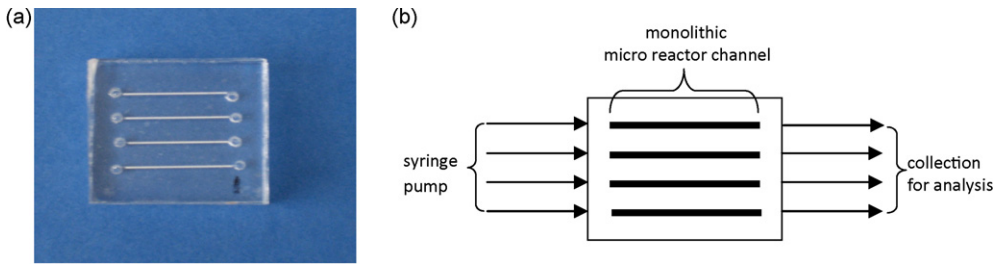


Fig. 1. (a) The monolithic micro-reactor developed for enzyme immobilisation in this work and (b) schematic illustrating how the system was utilised for biotransformation reactions where all four reactor channels were simultaneously used.

mixing and purged with nitrogen for 30 min. The micro-channels were then filled with the polymerisation solution and the areas where monolith formation was not desired were covered with an opaque tape. Polymerisation was initiated by placing the micro-reactors under a UV lamp (365 nm) and irradiated for 30 min. The epoxy derived monoliths were then washed with methanol and dried with nitrogen prior to use. Although straight reaction channels were used for this particular investigation, it must be noted that this protocol can be readily adapted for any channel pattern including those containing serpentines.

2.2. Preparation of L-aminoacylase (E.C. 3.5.1.14)

A starter culture of *E. coli* over-expressing the *T. litoralis* L-aminoacylase was grown overnight in Luria Bertani broth, containing ampicillin (100 mg l^{-1}), at 37 °C and at 200 rpm. This culture was used to inoculate 1 l flasks, containing media as above which were incubated at 37 °C and at 200 rpm. Gene expression was induced by the addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) and the cells were harvested 16 h after induction, by centrifugation at 18,000 × *g* for 30 min at 4 °C. A cell-free enzyme extract was prepared by resuspending the cell paste (10%, w/v) in 100 mM Tris–HCl pH 8.0 buffer. The cell suspension was then submitted to four cycles of 30 s pulses of sonication. The cell debris was removed by centrifugation at 12,000 × *g* for 15 min at 4 °C and this cell-free supernatant was used for all enzyme investigations.

2.3. Enzyme immobilisation and evaluation of immobilised enzymatic activity

The cell-free supernatant containing recombinant L-aminoacylase (E.C. 3.5.1.14) from the thermophilic archaeon, *T. litoralis*, was immobilised onto the monolithic channels *via* the primary amine group of the enzyme within the micro-reactors by passing a solution of L-aminoacylase in Tris–HCl buffer (1/100, v/v) through the channels using a syringe pump (Bioanalytical Systems Inc., MD-1001) at a flow rate of 1 μl min^{-1} for 3 h. Any unbound enzyme was then washed out of the micro-channels with Tris–HCl, pH 8.0 buffer for 30 min prior to use. Substrates were screened by dissolving them in 100 mM Tris–HCl buffer pH 8.0. The substrate solutions were charged into a 1 ml syringe and supplied to the monolithic micro-reactor using a microfluidic pump. The activity of the immobilised enzyme to each substrate was investigated at flow rates of 1–8 μl min^{-1}, at both room temperature and 50 °C. The initial biotransformations used 10 mM *N*-benzoyl-L-phenylalanine as the model substrate and the reaction products analysed by HPLC equipped with a 4.6 × 150 mm C18 BDS Hypersil column (Thermo Electron Corporation, UK) using 35% acetonitrile in water as the mobile phase at a flow rate of 2 ml min^{-1} at ambient temperature; the product and substrate peaks were detected by UV adsorption at 210 nm. Activities towards the *N*-protected amino acids were also determined by a modified Cd/Ninhydrin method previously reported [5]. The experiments were conducted in duplicate and where the result gave 100% conversion, the results were identical; error bars are shown for the other experiments (flow rates >5 μl min^{-1}) however in all cases the error was <2%.

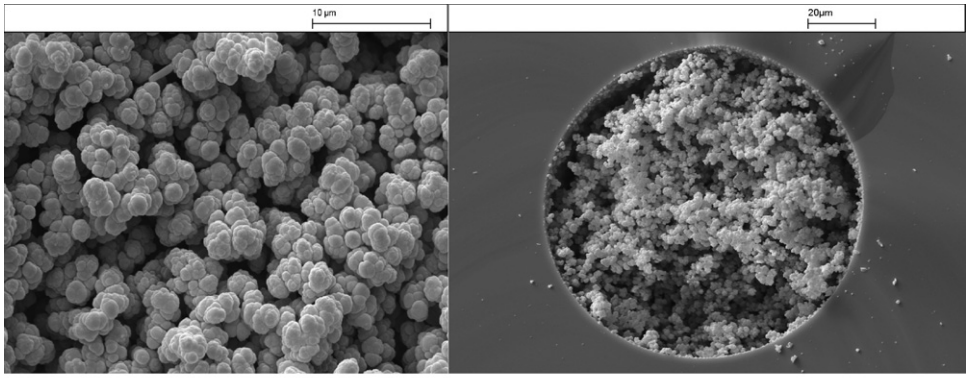
The enzyme specificity towards other substrates was also screened; *N*-acetyl-L-phenylalanine, *N*-chloroacetyl-L-phenylalanine, *N*-CBZ-L-phenylalanine, *N*-Boc-L-phenylalanine, *N*-acetyl-L-tryptophan, *N*-benzoyl-L-leucine, *N*-acetyl-L-tyrosine, *N*-benzoyl-L-threonine, *N*-acetyl-D,L-leucine, *N*-acetyl-L-methionine, *N*-acetyl-D,L-serine, *N*-acetyl-L-lysine and *N*-benzoyl-L-arginine. The reactions were carried out using 10 mM of each substrate in 100 mM Tris–HCl pH 8.0 buffer and run at a flow rate of 1 μl min^{-1} at room temperature. Although the enzyme's optimum temperature was found to be 85 °C, the preliminary experiment conducted in the developed micro-reactor suggested that the immobilised enzyme, with the monolithic micro-channels, could readily convert the most specific substrate (*N*-benzoyl-L-phenylalanine) (high enzyme activity could be obtained) at room temperature and a flow rate of 1 μl min^{-1}, see Section 3.1. Experiments on substrate screening were therefore carried out at these reaction conditions.

3. Results and discussion

3.1. Development of a micro-reactor for immobilised thermophilic L-aminoacylase from T. litoralis

The development of a reproducible monolith was challenging as there were several criteria that had to be considered. In the first instance the surface of the monolith needs to have an appropriate functional group onto which the enzyme could be anchored; an epoxy group (using glycidyl methacrylate as the monomer and ethylene dimethacrylate as the cross-linking agent) was found to enable efficient enzyme immobilisation to be achieved. The monolith was also required to have a high surface area for efficient immobilisation but, at the same time to have a high porosity to prevent the build-up of back pressure, especially during enzyme introduction; furthermore it needs to be formed in a reproducible manner. Attempts to make highly porous monoliths with low back pressure were made by (a) adjusting the monomer to porogenic mixture ratios and (b) varying the alcohol (1-propanol, 1-butanol, heptanol, octanol, decanol, dodecanol and cyclohexanol) employed in the porogenic mixture (results not shown here). The monoliths with least back pressure were prepared using cyclohexanol and dodecanol (4:1) as the porogenic mixture.

The SEM images (Fig. 2) show the internal morphology of a prepared monolith which demonstrates that the porous polymer was homogeneous across the entire monolith. The absence of voids between the channel wall and the monolith also indicates that the monoliths were covalently bound to the surface of the glass channels. It is important that the monolith is bound to the walls so that it would not be pumped out of the micro-reactor during the flow reaction and/or the chemicals would not by-pass the immobilised enzyme.



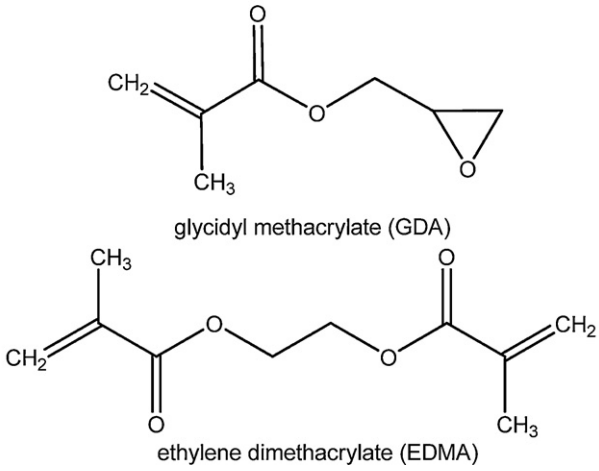


Fig. 2. SEM images of the synthesised monoliths formed within micro-reactors.

Prior to immobilisation of the enzyme, flow studies were conducted and it was found that flow rates up to 8 μl min^{-1} could be utilised within these systems however applying higher flow rates led to high back pressure, resulting leaks at the inter-connections.

Once the monolith was successfully produced, and evaluated as described, the thermophilic enzyme L-aminoacylase was then immobilised *in situ*. As described previously this was achieved by pumping the enzyme through the micro-reactor channels and allowing it to bind to the monolith *via* reaction between the amino groups of the enzyme and the epoxy terminal groups on the surface of the monolith. It should be emphasised that it was not possible to measure the enzyme loading because the amount of enzyme was too small to quantify and therefore the activity of the enzyme on the monolith was monitored indirectly *via* the conversion of the substrate into the desired product. The success of this methodology was initially investigated by measuring its ability to convert the model substrate, 10 mM *N*-benzoyl-L-phenylalanine to L-phenylalanine. The reaction was carried out in Tris–HCl pH 8.0 buffer where highest activity was observed for the free enzyme [5] and the enzyme activity was investigated at flow rates of 1–8 μl min^{-1} (Fig. 3). Although this enzyme is known to have optimal activity at 85 °C [5,8], it was found that complete substrate conversion could be obtained in the micro-reactor at room temperature ($\leq$4 μl min^{-1}); at higher flow rates the substrate conversion decreased. Since the enzyme is thermophilic, the reaction temperature can be increased above ambient conditions. The operating window in the micro-reactor was therefore investigated by increasing the reaction temperature to 50 °C and increasing the flow rate up to 8 μl min^{-1}; the highest flow rate possible in the monolith without having back pressure problems. At this higher temperature, 100% conversion of the substrate was obtained despite the increase in flow rate. One long term goal is therefore to prepare more porous monoliths to enable higher flow rates to be used which would allow a higher product throughput to be achieved. It must be noted however that under the aforementioned conditions all monoliths prepared were found to be reproducible with respect to flow rate and substrate conversion.

As the idea of enzyme immobilisation is to generate enzymes with higher operational stability and improve the storage lifetime, the immobilised enzyme was subsequently subjected to a stability test both during continuous use and when stored in buffer at 4 °C. When the enzyme was immobilised onto the monolithic micro-reactor and operated at room temperature it produced reproducible substrate conversion over 120 h (continuous use), with no loss in activity. When the micro-reactor was operated at a

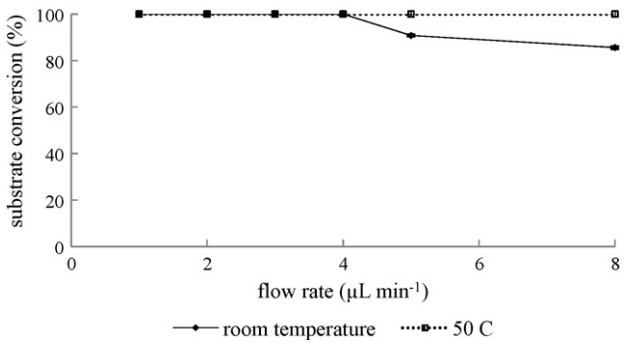
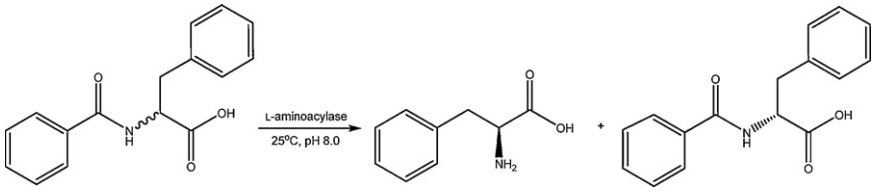


Fig. 3. Effect of flow rate on the activity of the enzyme in converting 10 mM. *N*-Benzoyl-L-phenylalanine in 100 mM Tris–HCl buffer pH 8.0 at room temperature and 50 °C.



Scheme 2. L-Aminoacylase catalyses the conversion of *N*-benzoyl-L-phenylalanine to L-phenylalanine while it is inactive in cleaving the D-form.

higher temperature (50 °C) it showed no activity loss after 50 h (the reaction was terminated after this point), confirming the enzymes stability at this temperature. These results indicate that the continuous reaction system consisting of the enzyme immobilised onto the monolithic micro-channels is a suitable tool for further development.

3.2. Utilising the developed micro-reactor for substrate screening

Having developed a successful micro-reaction system where the thermophilic L-aminoacylase from *T. litoralis* was immobilised onto the monolithic micro-channels and 100% conversion of 10 mM *N*-benzoyl-L-phenylalanine to L-phenylalanine was achieved at room temperature, the next step was to investigate the use of the micro-reactor as a screening tool for other substrates. The first experiment looked at the enzyme stereospecificity following its immobilisation onto the monolithic micro-channels, using a racemic substrate of 10 mM *N*-benzoyl-D,L-phenylalanine. In this biotransformation the enzyme only cleaves the L-form while leaving D-form unreacted, Scheme 2. The maximum substrate conversion was found to be 50% as predicted (Table 1) confirming the enzyme's stereospecificity.

To confirm that the immobilised enzyme was working in a reliable manner within the micro-reactor environment, substrate screening was initially carried out on a set of substrates previously studied [5,8]. In the first instance the investigation was focused on determining the most preferred *N*-protecting group for the amino acid, this protecting group was subsequently used to study what other amino acids could be converted by the enzyme.

In previously published work, the enzyme has shown greater specificity towards benzoyl- and chloroacetyl-, rather than acetyl- or other *N*-protecting groups [5]. In order to determine whether the immobilised enzyme retained its selectivity towards potential *N*-protecting groups, it was tested with substrates containing the same amino acid residue, Phe, with different *N*-protecting groups (benzoyl-, chloroacetyl-, acetyl-, Boc- and Cbz-). The enzymes ability to convert the potential substrates was measured at room temperature, Table 1.

Table 1
Activity of immobilised L-aminoacylase towards its potential substrates, recorded using the micro-reactor operated at room temperature with a flow rate of 1 μl min^{-1}. All substrates were screened at the concentration of 10 mM.

Substrate	Substrate conversion (%)
N-Benzoyl-D,L-Phe	50.0
N-Benzoyl-L-Phe	100, 100.0[a]
N-Chloroacetyl-L-Phe	100, 90.1[a]
N-Acetyl-L-Phe	100, 66.1[a]
N-*t*-BOC-L-Phe	40.9
N-CBZ-L-Phe	30.9
N-Benzoyl-L-Thr	68.3
N-Benzoyl-L-Leu	52.2
N-Acetyl-D,L-Ser	25.6[b]
N-Benzoyl-L-Arg	43.6
N-Acetyl-D,L-Leu	20.5[b]
N-Acetyl-L-Met	38.7
N-Acetyl-L-Tyr	33.3
N-Acetyl-L-Trp	7.0
N-Acetyl-L-Lys	0

[a] Substrate conversions observed at 2 μl min^{-1}.
[b] Substrate conversions out of a maximum of 50%.

At 1 μl min^{-1}, *N*-benzoyl-L-Phe, *N*-chloroacetyl-L-Phe and *N*-acetyl-L-Phe were readily hydrolysed by the enzyme (100% conversion), whereas lower conversions were found for Boc- and Cbz-protecting groups; 40.9 and 30.9%, respectively. To study in more detail, the enzyme specificity towards benzoyl-, chloroacetyl- and acetyl-groups the reaction flow rate was increased to 2 μl min^{-1}. At this increased flow rate, the enzyme remained very active towards *N*-benzoyl- (100% conversion) but lower substrate conversions were obtained for *N*-chloroacetyl- (90.1%) and *N*-acetyl- (66.1%). The order of preferred *N*-protecting groups can therefore be summarised as follows: benzoyl > chloroacetyl > acetyl > Boc > Cbz. Generally, the immobilised L-aminoacylase behaved similarly to the free L-aminoacylase but it differed in that it was more active towards Boc- than CBZ-protecting groups. This may be due to the fact that the CBZ-group is more sterically demanding than the Boc-group, preventing it from accessing all of the immobilised enzyme.

Additionally, substrate specificity was investigated for different amino acids. Having found that the most specific protecting group for the thermophilic L-aminoacylase was benzoyl-, other amino acids with this protecting group which have not been reported before were chosen for the investigation, *i.e.* threonine, leucine and arginine. The order of preferred amino acids was found to be Phe > Thr > Leu > Arg. The results confirm the enzymes preference for Phe over Arg regardless of the protecting group of the substrates (either acetyl or benzoyl; much higher activity of Phe over Arg was reported when the study was carried out using *N*-acetyl protecting group on both amino acids) [5]. It is interesting to note that despite poor activity towards *N*-acetyl-L-arginine, the enzymes ability to convert *N*-benzoyl-L-arginine was moderate (43.6%).

Substrates containing other amino acids with *N*-acetyl-protecting group were also examined both on previously reported substrates (Phe, Met and Trp) and other new substrates (Ser, Leu and Tyr). Substrate screening over serine and leucine were performed using the racemic form, where only 50% maximum substrate conversion could be expected. The order of preference was found to be Phe ≫ Ser > Leu > Met > Tyr > Trp; the enzyme was nevertheless found to be inactive towards lysine. The immobilised enzyme behaved in a similar manner to the free enzyme towards Phe, Met and Trp. Generally, the enzyme appears to prefer hydrophobic and uncharged amino acids, with a similar trend observed when taking *N*-benzoyl- or *N*-acetyl substrates into consideration. For instance, among the *N*-benzoyl substrates, the enzyme was least specific towards arginine which is hydrophilic, polar and charged at the operating pH 8.0. Additionally, the enzyme was incapable of cleaving *N*-acetyl-L-lysine which is also hydrophilic, polar and charged at the operating pH.

After being employed to screen various substrates, the micro-reactor was again subjected to the most preferred substrate, *N*-benzoyl-L-Phe, in order to check whether the enzyme was still fully active; as expected its ability to convert the preferred substrate to L-phenylalanine was found to be retained.

In summary, the use of an immobilised enzyme within monolithic micro-channels has proved to allow rapid screening in a

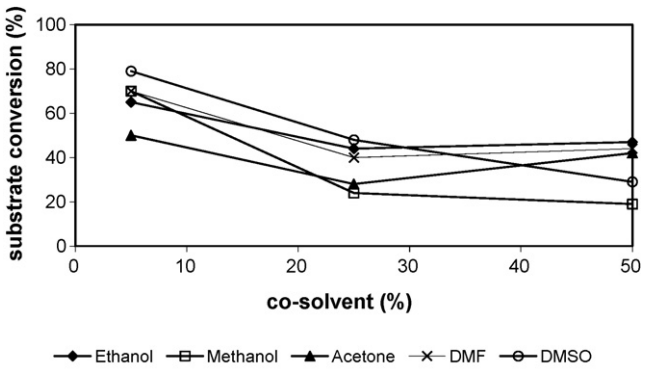


Fig. 4. Effect of solvents on substrate conversion; the reaction was performed with 10 mM *N*-benzoyl-L-phenylalanine at room temperature for a period of 1 h using a flow rate of 1 μl min^{-1}.

reliable manner compared to utilising the free enzyme in a conventional batch system. The micro-reactor experiments were analysed every hour in order to evaluate the reaction efficiency over time, however in screening experiments it was possible to conduct individual experiments within 10 min, the limitation being the sensitivity of the analytical method used for quantification. The major advantages include the lower quantity of reagent required, the reuseable nature of the enzyme in the micro-reactor and the milder reaction conditions introduced by the use of micro-reactors.

3.3. Use of the developed micro-reactor system in organic solvents

Many biotransformation reactions need to be carried out in organic solvents due to the insolubility of the substrates being used and where inactivation is observed for homogeneous biocatalysts, enzyme immobilisation onto solid supports often helps to stabilise enzymes for use under these conditions. Rapid inactivation of L-aminoacylase from *T. litoralis* in the presence of 50% organic solvents at 60 °C was previously reported [5] and the presence of 10% *N*,*N*-dimethylformamide (DMF) was also found to have caused more than a 70% decrease in stability of aminoacylase from hog kidney after 50 min at 25 °C [24].

Evaluation of enzyme conversion of 10 mM *N*-benzoyl-L-Phe in Tris–HCl pH 8.0 buffer, the most favourable substrate, in the presence of organic solvents was carried out in the developed micro-reactor using a flow rate of 1 μl min^{-1} and the reaction was performed at room temperature (the reaction conditions where 100% substrate conversion was found in the aqueous system). To evaluate the effect of organic solvents on the debenzoylation of *N*-benzoyl-L-Phe, the following solvents were studied at varying proportions (5–50%, v/v); DMF, dimethylsulfoxide (DMSO), ethanol, methanol and acetone.

At least 50% decrease in enzyme activity generally occurred in the presence of 50% organic solvents and the effect was less pronounced when lower quantities of solvent were used, Fig. 4. This can be explained by the fact that the more hydrophobic the solvent molecules, the less favourable binding of the substrate to the active site, thereby reducing enzyme activity. However the use of 5% DMSO, enough to dissolve the substrate, resulted in only *ca.* 20% decrease in enzyme activity. The combination of a small amount of DMSO and higher reaction temperature suggests the potential of employing the enzyme in a system where substrate solubility in Tris–buffer is an issue. It needs to be clarified that when acetone was used as the solvent the enzyme was inactivated, however when other solvents were used (ethanol, methanol, DMF and DMSO) this was not a problem.

4. Conclusions

A micro-reactor containing polymer monoliths formed inside the micro-channels was developed for biotransformation reactions catalysed by the thermophilic L-aminoacylase from *T. litoralis*. The micro-reactor proved to be a useful tool for high throughput screening of potential substrates, where individual substrates could be evaluated in approximately 10 min after reactor characterisation. This robust system was reliable (as we determined that enzyme activity was retained for 50 h even at 50 °C) and it also reduced enzyme consumption with high stability under both operational and storage conditions. Additionally, it could be operated at room temperature to yield similar enzyme activities for some preferred substrates (*N*-benzoyl-L-Phe and *N*-chloroacetyl-L-Phe). The operating temperature could also be increased for less preferred substrates to gain higher enzyme activities. Clearly using the reactor at room temperature was advantageous as we had demonstrated that the activity was retained for a longer period of time. The stability of the enzyme micro-reactor system as well as the enzyme activity in the appropriate solvent to aid substrate solubility suggested the potential use of this system for high throughput biotransformations.

The system developed was also useful for screening new potential substrates with a *N*-benzoyl-protecting group which was not the most specific substrate for L-aminoacylase from other sources. Further system development will be directed towards improving the porosity of the monoliths, with a view to a reduction in back pressure, since this should allow higher flow rates and subsequently a higher throughput to be attained.

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