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Development and use of a microreactor for the study of biotransformations by a cross-linked γ-lactamase enzyme

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Abstract

A (+)-γ-lactamase was precipitated, cross-linked and the resulting solid crushed prior to immobilization within a capillary column micro reactor. The micro reactor was subsequently used to study enzyme stability, activity, kinetics and substrate specificity. The thermophilic (+)-γ-lactamase retained 100 % of its initial activity at the assay temperature, 80 °C, for 6 h and retained 52 % activity after 10 h, indicating the advantage of immobilization. This high stability of the immobilized enzyme provided the advantage that it could be utilized to screen many compounds in the microreactor system. This advantage overcame the fact that the immobilization process affected enzyme kinetics and activity, which was reduced (by 70%) compared to the free enzyme. In general, the enzyme displayed similar substrate specificity to that found in a previous study for the free enzyme, however, enhanced activity was seen towards one substrate, acrylamide. The system developed correlates well with the free enzyme in batch assay and indicates the suitability of the system for enzyme substrate screening, allowing a significant reduction in cost, due to the reduced amounts of enzyme, substrates and other assay constituents required.

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1. Introduction

In recent times, biocatalysis has received much attention with regard to the synthesis of precursors and intermediates in the production of high value chemicals. The use of enzymes in the production of chiral intermediates for pharmaceuticals, along with the synthesis of chiral bulk, fine chemicals and active pharmaceuticals have been the subject of numerous reviews[1-3]. The reasons for interest in enzymes include their substrate specificity and ability to generate chiral products in high purity, which is particularly advantageous when specific high-value products are required and chemical synthesis has failed to produce a similar product.

The discovery of potential industrial biocatalysts is often limited by a number of factors, such as the potential cost and instability of the enzymes of interest. Screening of potential biocatalysts is usually performed in batch reactions, which can prove expensive when enzymes serve a single-use purpose. However, their immobilization reduces the cost of production considerably because, rather than being lost at the end of the biocatalytic process, the enzyme can be recycled and used again. Immobilization has also been shown, previously, to increase enzyme stability [4-6].

Microfluidic reactors offer a solution to the costly nature of screening enzymes for activity on a range of substrates as they afford a significant reduction in reagent quantities required, due to their reduced scale when compared with batch reaction systems, thus reducing the cost of screening a range of substrates. Originally developed for chemical applications, microfluidic reactors offer advantages which

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include a reduction in cost, greater control of reaction parameters, hazard reduction and potential for scale-up [7].

The application of enzymes as biocatalysts in microfluidic reactors has been previously demonstrated and a number of different approaches have been studied [8, 9]. In order to limit the cost of enzyme use in microfluidic reactors, enzymes are often immobilized. A number of immobilization methods frequently used in larger reactors, such as alginate-, polyacrylamide- and sepharose-based gels are unsuitable for use in microfluidic reactors, due to their propensity to generate prohibitive back pressures. Among the methods of immobilization used, monoliths have proved quite successful, particularly when used with trypsin [10-15]. Peterson *et al*. found that tryptic digests performed by monolith-bound trypsin in microfluidic reactors exhibited 67 % sequence coverage of myoglobin, yielding 102 of 153 possible peptide fragments [9]. The disadvantage of the monolith systems is that high back pressures can occur when pumping the substrate through the monolith. An alternative approach has therefore been to crosslink the enzyme to the microfluidic reactor surface which has met with some success for analytical separations. Honda *et al*. cross-linked L-aminoacylase to PTFE, allowing substrate flow over the surface to allow conversion [16], while Thomsen *et al*. cross-linked β-galactosidase to an amino-silanized microfluidic reactor [17]. This approach does not, however, provide the high loading of enzyme required for synthetic conversions.

The aim of this work was develop a robust screening method to study biotransformations using (+)-γ-lactamase enzyme. This activity has been demonstrated to be important to produce (-) γ-lactam which is an important building

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block for the synthesis of carbocyclic nucleosides which have potent anti-viral activity due to their inhibitory effect on viral reverse transcriptase [18-20]. In previous work a (+)-γ-lactamase enzyme from *Sulfolobus solfataricus* MT4 had been cloned and overexpressed in *Escherichia coli* DH5 [21] and was shown to have a preference for (+)-γ-lactam, while exhibiting activity towards a range of amides. This paper describes an effective method where a cross-linked γ-lactamase, obtained by precipitation and chemical cross-linking, has been packed into a channel to give an efficient enzyme micro-reactor with low back pressure.

2. Materials and Methods

2.1 Chemicals

Chemicals were purchased from the sources indicated and were used as supplied; acrylamide (97%, Aldrich), acetamide (98%, Sigma), *m*-aminobenzamide (99%, Sigma), *p*-aminobenzamide (98%, Aldrich), *m*-aminobenzoic acid (98 %, Aldrich), benzamide (99 %, Aldrich), benzoic acid (99 %, Aldrich), butyramide (98 %, Fluka), isobutyramide (99 %, Aldrich), propionamide (97 %, Aldrich), formamide (98 %, Avocado), (+/-)-γ-lactam (98 %, Aldrich), nicotinamide (98 %, Sigma), (R)-(+)-lactamide (97 %, Aldrich), *m*-toluamide (99 %, Aldrich), *p*-toluamide (99 %, Aldrich), *m*-toluic acid (99 %, Fluka), *p*-toluic acid (98 %, Fluka), nicotinic acid (99 %, Sigma-Aldrich), formaldehyde (40 % solution, BDH), ammonium sulphate (≥99 %, Sigma), bicinchoninic acid (Sigma), bovine serum albumin (>98 %, BSA) (Sigma). Methanol and acetonitrile used as HPLC mobile phase were of HPLC grade and purchased from Fisher Scientific.

2.2 Preparation of (+)-γ-lactamase

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2.2.1 Enzyme Solution

An overnight culture of *Escherichia coli* harbouring the (+)-γ-lactamase gene was grown in Luria Bertani broth containing ampicillin (100 mg L^{-1}) at 37 ºC and 200 rpm. This overnight culture was used to inoculate 1 L cultures and expression of the gene was induced by addition of IPTG (1 mM) and incubation for a further 3 h. The cells were harvested by centrifugation at 12,000 *g* for 20 min at 4 ºC and resuspended in Tris-HCl, pH 8.0 (10 % w/v). The cells were disrupted by four cycles of 30 s of sonication. The cell debris was precipitated out of suspension by centrifugation at 18,000 *g* for 30 min at 4 ºC. This cell-free supernatant was used for all investigations.

2.2.2 Cross-Linked Enzyme

The method used was based on the method of Cao *et al*. [22]. A cell-free enzyme solution was precipitated with ammonium sulfate (80 % saturation), on ice with stirring. The precipitate was cross-linked by the addition of formaldehyde (1 % v/v) and the suspension was left to stir on ice for 1.5 h. The precipitate was then filtered using Whatman No. 1 filter paper and washed several times with water to remove all traces of formaldehyde. The cross-linked enzyme was dried in a desiccator, at which point the cross-linked enzyme was ready for use in the micro reactor.

2.3 Protein Determination

Protein concentration was determined by a modification of the bicinchoninic acid method for protein determination supplied by Sigma-Aldrich. BSA was used as a standard in the range 0-1 mg mL^{-1}.

2.4 Microfluidic Enzyme Reactor

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The microfluidic enzyme reactors used consisted of capillary tubes with a frit at one end (length = 6.5 cm and i.d. = 1.2×10^{-3} m, BLAUBRAND®). Detail for the preparation of the frit is found in [23]. The dried cross-linked (+)-γ-lactamase was mixed with controlled pore glass (120-200 mesh, 500 Å nominal pore diameter) in the ratio 1:1 and packed in a fritted capillary column.

2.5 Enzyme Assays

2.5.1 Standard (+)-γ-lactamase Assay:

(+)-γ-Lactamase activity was determined by incubating the enzyme in a 1 mL assay, containing 10 mM benzamide in 10 mM KH_2PO_4/K_2HPO_4 (pH 7.0) for 10 min at 80 °C. Analysis was performed by high performance liquid chromatography (HPLC). The reaction was terminated by adding 100 μL to 900 μL of 50:50 methanol: KH_2PO_4/K_2HPO_4 (pH 7.0), the mobile phase. Samples were injected onto a BDS C_{18} Hypersil® reversed-phase column (Phenomenex, UK) and a mobile phase flow rate of 1 mL min^{-1} employed. The eluent was monitored at 225 nm. One unit of enzyme activity (U) is defined as the amount of enzyme that converts 1 μmole of benzamide to benzoic acid in 1 min at a given temperature.

2.5.2 Micro reactor Enzyme Assay

A solution of 10 mM benzamide in KH_2PO_4/K_2HPO_4 (pH 7.0) was passed through the enzyme bed using a syringe pump at flow rates between 1-5 μL min^{-1}. Enzyme activity was initially investigated by monitoring the conversion of benzamide to benzoic acid at temperatures between 30-80 °C. Substrate screening was also carried out on a number of other substrates. Substrates and reaction products were detected by HPLC equipped with a BDS C_{18} Hypersil® column.

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For most assays, detection was performed at 225 nm with a flow rate of 1 mL min^{-1} and 50:50 methanol: KH_2PO_4/K_2HPO_4 (pH 7.0) used as the mobile phase.

HPLC conditions to detect nicotinamide and nicotinamic acid were 216 nm, 1 mL min^{-1} and 20:20:60 methanol: acetonitrile: water.

Other aliphatic substrates and aliphatic acids were detected at 210 nm, with the same column and mobile phase.

The formation of benzoyl hydrazide was investigated by reacting 10 mM benzamide with 400 mM hydrazine in KH_2PO_4/K_2HPO_4 (pH 8.5) at 80 ºC and a flow rate of 1 μL min^{-1}. The reaction was monitored by HPLC operating at 225 nm with a flow rate of 1 mL min^{-1} and 50:50 methanol: KH_2PO_4/K_2HPO_4 (pH 7.0) was used as the mobile phase.

3. Results and discussion

3.1 Immobilization and optimization of activity of cross-linked (+)-γ-lactamase in a capillary column micro reactor

Cross-linked (+)-γ-lactamase (Figure 1) was dried and crushed and packed in a ratio of 1:1 with controlled pore glass in silica-fritted capillary tubes. The enzyme was initially packed in the capillary tubes without controlled pore glass but the levels of back pressure developed were prohibitive to the systems operation (results not shown). The cross-linked protein concentration was calculated based on the difference between the protein concentration prior to and after precipitation and cross-linking and indicated that the dry weight of the cross-linked protein was equal to the predicted protein content, based on these calculations. The cross-linked (+)-γ-lactamase protein sample was from a crude protein mixture where it was over-

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expressed to represent 5% of the total protein, as based on observation on a SDS-PAGE gel. Therefore it was not possible to determine the absolute concentration of the enzyme. The total amount of protein in the micro-reactor column was 5mg protein as determined by the bicinchoninic acid assay as described in Materials and Methods.

Once the enzyme was immobilized it was tested for activity, by measuring its conversion of the aromatic amidase substrate, benzamide (10 mM). Enzyme activity was measured, using a flow rate of 1 μL min^{-1}, at different temperatures (Figure 2). Enzyme activity was found to be optimal at 80 ºC, a result similar to that found previously [21]. All further experiments were carried out at this temperature, unless otherwise indicated. Despite the enzyme being of thermophilic origin, it was still necessary to investigate its ability to retain its activity in this system at the reaction temperature.

3.2 Performance of (+)-γ-lactamase in microfluidic reactor

The performance of the immobilized enzyme micro reactor was monitored by continuous operation of the micro reactor at 80 °C, using a benzamide concentration of 10 mM. Enzyme activity was found to be retained at 100 % conversion for 6 h of continuous flow, after which there was a reduction in activity, with 52 % conversion after 10 h (Figure 3), indicating the enzymes ability to retain activity for a significant time at the reaction temperature. This is a significant improvement when compared with the free enzyme, which began to display a reduction in activity after 1 h at 80 °C. After 6h, when the immobilized enzyme began to display a loss of activity, the free enzyme activity was less than 20% of the original activity observed. The

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thermal stability results suggest that the cross-linked γ-lactamase has potential for further development, especially in biotransformations where a higher temperature can be advantageous in increasing substrate solubility and rate of reaction.

Enzyme activity was also measured at a number of flow rates (0-10 μL min^{-1}), to determine the effect of flow rate on activity (Figure 4). The conversion of substrate, 2.5, 5.0, 10 mM benzamide, at a number of flow rates up to 50 μL min^{-1} was monitored (all results not shown). While 100 % conversion was maintained at all concentrations at a flow rate of 1 μL min^{-1} at 80 °C, conversion was reduced at the lower temperatures of 60 °C and 70 °C (Figure 4a). At shorter residence times, where faster flow rates (≥ 2 μL min^{-1}) were used, conversions were also reduced (Figure 4b).

3.3 Enzyme substrate screening

In previous studies free (+)-γ-lactamase was found to convert only (+)-γ-lactam in a racemic mix [21]. The enzyme micro reactor also converted 50.0 % of racemic γ-lactam, confirming the retention of its stereospecificity after immobilization. The enzyme was screened for activity against a range of aliphatic substrates (Table 1). The enzyme was not active towards formamide and acetamide, whose chains appear to be too short for good interaction. Hydrophobic interactions with substrates consisting of aliphatic carbon chains were minimally achieved. Activity towards propionamide (16.0 %) was half that observed for butyramide (33.4 %). The enzyme displayed a preference for branched chains, as indicated by its preference for isobutyramide (58.7 %) over butyramide. Substrate specificity of the immobilized (+)-γ-lactamase was also investigated for other aromatic substrates, *i.e. m*-toluamide,

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p-toluamide, *m*-aminobenzamide, *p*-aminobenzamide and nicotinamide using the optimum reaction conditions as observed from the use of benzamide as a substrate. Among the other aromatic amides tested, *m*-toluamide and *p*-toluamide were readily hydrolyzed (100.0 %) whereas lower activities were observed for *m*-aminobenzamide (24.3 %) and *p*-aminobenzamide (11.0 %). While the aromatic amidase from *Pseudomonas sp*. GDI 211 exhibited activity on nicotinamide, the immobilized (+)-γ-lactamase was not found to be active towards nicotinamide [24]. This lack of enzyme activity towards nicotinamide suggests the negative effect of the substitution of one carbon of the ring with nitrogen. Similar behaviour was reported in other work involving this enzyme, reported as a recombinant amidase, where the hydrophobic substituent CH_3 in the aromatic ring had positive effect and the hydrophilic substituent NH_2 negatively affected the activity, independently of the position of substitution with respect to the carboxamide group [25]. To discriminate between the substrates that exhibited 100 % conversion, the cross-linked enzyme was assayed at a faster flow rate of 2 μL min^{-1}, allowing a residence time of 31.5 min. Once the micro reactor flow rate was increased, it was found that substrate preference was in the following order (+/-)-γ-lactam > (R)-(+)-lactamide > benzamide > *m*-toluamide > *p*-toluamide, similar to the original investigation with the free enzyme [21]. The enzymes conversion of toluamide and aminobenzamide indicated that the enzyme had a preference for *m*- over *p*-substituted substrates. Another amidase enzyme, isolated from *Pseudonocardia thermophila*, also exhibited broad specificity, but was selective for aliphatic amides such as propionamide and acrylamide and the *S*-form of 2-phenylpropionamide [26], a feature also observed in

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Sulfolobus tokodaii strain 7 [27] and *Sulfolobus solfataricus* MT4 [25, 28], which indicates that the enzyme under investigation in this work has the potential to favour either (*S*)- or (*R*)-forms of different substrates, since it has been shown to be selective for (*R*)-(+)-lactamide and the (*S*)-form of 2-phenylpropionamide. In contrast to the previous study, the enzyme was moderately specific toward acrylamide (39.4 %) within the miniaturised flow reactor. This increased activity may be attributable to increased contact between enzyme and substrate in the micro reactor set-up.

Although the enzyme micro reactor was assayed at its optimum temperature, the enzyme was still active at lower temperatures which could, therefore, be used if required.

The immobilized (+)-γ-lactamase was also examined for acyl transfer activity, a property of amidases first described in *Rhodococcus rhodochrous* J1 by Kobayashi *et al*. [29]. In the presence of hydrazine, it was found to be capable of producing 100% yield of benzoyl hydrazide with no benzoic acid by-product. This also correlated well with previous work using this clone [21].

3.4 Comparison of activity of immobilized and soluble enzyme

3.4.1 Enzyme activity studies

Enzyme activity was measured with 10 mM benzamide in phosphate buffer, pH 7.0. Specific activity was calculated and cross-linked (+)-γ-lactamase was found to have an activity of 0.122 U mg^{-1} when assayed at 80 ºC, with a residence time of 63 min. Soluble (+)-γ-lactamase activity was found to be 0.400 U mg^{-1}, which indicates a 69.5 % reduction in enzyme activity upon immobilization. Although the free enzyme

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activity is lower than the activity reported previously [21, 25], it can be explained by the fact that the enzyme preparation was a crude cell extract, which had not undergone any purification. In these papers, activity towards benzamide was 7.4 U mg^{-1} and 13.8 U mg^{-1}, respectively, at 85 ºC. While purification may have increased activity, it may also have resulted in a reduction in the proportion of enzyme activity retained after immobilization, since the presence of a number of other proteins may have had a protective effect towards the enzyme. The reduction in enzyme activity upon immobilization was quite significant and would indicate the severity of the immobilization procedure. Whereas there was a 69.5 % drop in amidase specific activity in microfluidic reactors, compared with free enzyme, a 40 % reduction was observed with penicillin acylase when it was immobilized in a similar manner [22]. It is not unusual to find a reduction in activity upon immobilization [30, 31] and quite often the extent is an enzyme-specific factor. This drop in activity was of less importance than the stability of the immobilized enzyme in micro reactors where 100% conversion is being achieved.

3.4.2 Enzyme kinetics studies

In order to further investigate the effects of immobilization on enzyme activity, a study of enzyme kinetics was carried out. Conversion of the model substrate, benzamide, by micro reactor-immobilized cross-linked protein was monitored at 80 ºC, with a flow rate of 21.5 μL min^{-1} and a residence time of 2 min, over a range of substrate concentrations, 0.5, 1.0, 1.5, 2.0 and 2.5 mM. The kinetic constants, K_m and V_{max}, were determined to be 1.89 mM and 0.22 mM min^{-1}, respectively. It is not

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possible to determine the K_{cat} of the enzyme since the exact concentration of enzyme in the cross-linked protein cannot be calculated (see section 3.1).

The kinetic constants of the free enzyme, in solution, were also determined for comparison. The enzyme was also assayed at 80 ºC and the K_m and V_{max} were determined to be 0.49 mM and 1.40 mM min^{-1}, a K_m of 0.3 mM was previously reported by d'Abusco *et al*. [25], they did not indicate the enzymes V_{max}. These results suggest that there was a reduction in enzyme affinity and maximum rate upon immobilization. They also suggest that immobilization caused reduced enzyme efficiency, probably the result of its exposure to formaldehyde during the immobilization process, which was also indicated by the reduction in enzyme specific activity. A similar effect was observed when laccase and chloroperoxidase CLECs were prepared by using glutaraldehyde to cross-link the laccase and chloroperoxidase crystals [32, 33]. While there was a significant reduction in enzyme activity, this did not prevent its investigation as a substrate screening tool, since the micro reactor system still required significantly lower quantities of enzyme and substrates than batch screening would require and correlated well with previous batch screening.

4. Conclusions

A thermophilic (+)-γ-lactamase has been immobilized in the form of a cross-linked protein mixture and the immobilized enzyme maintained thermal stability at its optimum temperature, 80 °C for 6 h, allowing extensive screening using one microfluidic enzyme reactor. Screening of potential substrates employing

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immobilized (+)-γ-lactamase in a miniaturised flow reactor can be achieved in a shorter period of time and requires significantly less substrate than conventional batch enzyme assays. While (+)-γ-lactam was the preferred substrate, the immobilized enzyme readily hydrolyzed a number of other aromatic amides, *i.e.* benzamide, *p*-toluamide and *m*-toluamide and exhibited an ability to produce acid hydrazides.

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Legend of Figures

Figure 1. SEM Image of cross-linked enzyme

Figure 2. Effect of temperature on substrate conversion by cross-linked enzyme

Figure 3. Enzyme activity retention at optimum reaction temperature by (○) free enzyme and (●) cross-linked enzyme.

Figure 4. Effect of flow rate on substrate conversion at (a) different reaction temperatures (●) 80 ºC, (○) 70 ºC, (▼) 60 ºC and (b) different concentrations at 80 °C. (●) 2.5 mM, (○) 5.0 mM, (▼) 10.0 mM

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Table 1. Substrates used by (+)-γ-lactamase. Substrate concentration of 10 mM was used, at a flow rate of 1 μL min^{-1}, at 80°C

*Substrate conversions observed at a flow rate of 2 μl min^{-1}

**Substrate conversions (out of a maximum of 50%)

Substrate	Substrate conversion (%)
(+/-)-γ-Lactam**	50.0 (50.0)*
(*R*)-(+)-Lactamide	100 (85.0)*
Benzamide	100 (61.1)*
m-Toluamide	100 (56.1)*
p-Toluamide	100 (41.0)*
Isobutyramide	58.7
Acrylamide	39.4
Butyramide	33.4
m-Aminobenzamide	24.3
Propionamide	16.0
p-Aminobenzamide	11.0
Nicotinamide	0
Acetamide	0
Formamide	0

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Figure 1

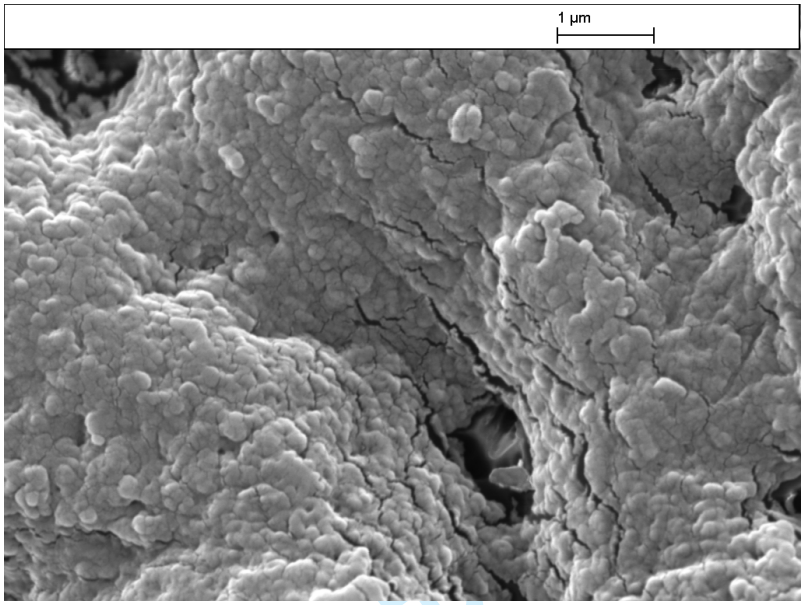

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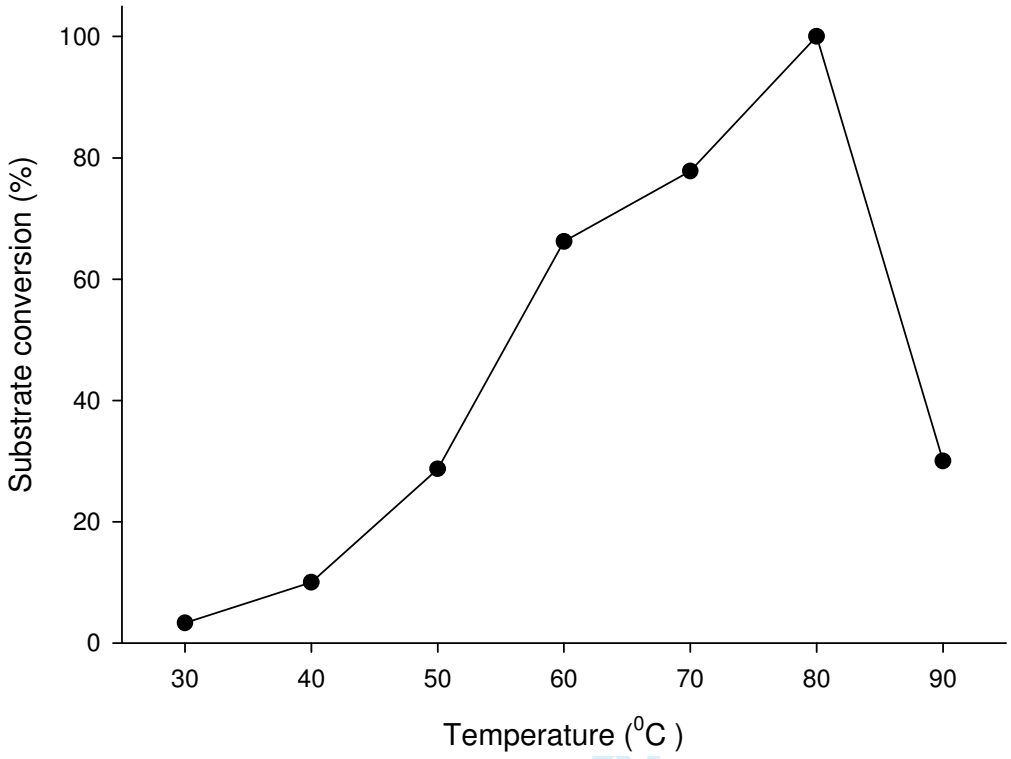
Figure 2.


Substrate conversion (%)
0
20
40
60
80
100
30
40
50
60
70
80
90
Temperature (^{0}C)

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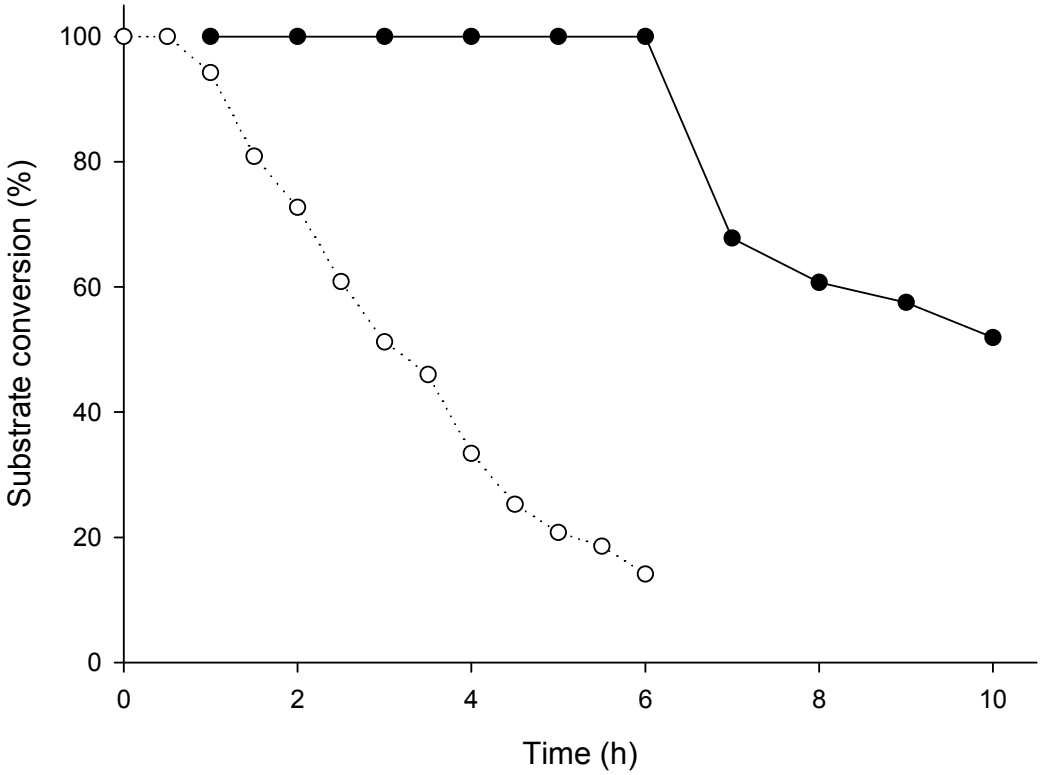

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Figure 4 (a).

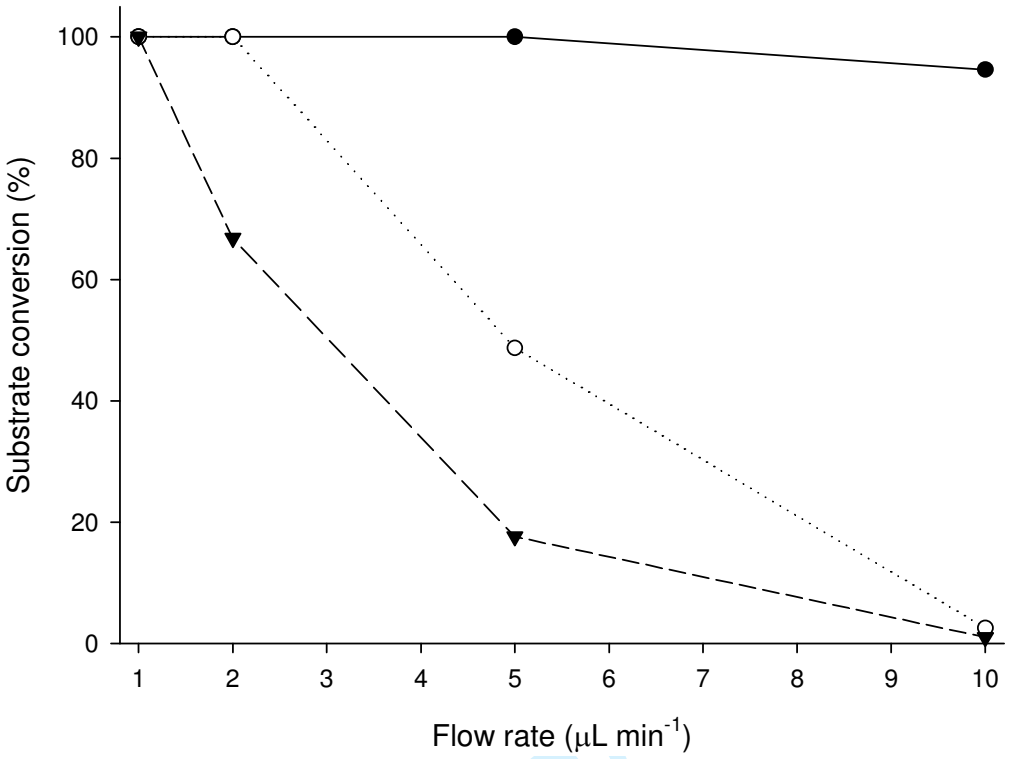

Figure 4 (b).

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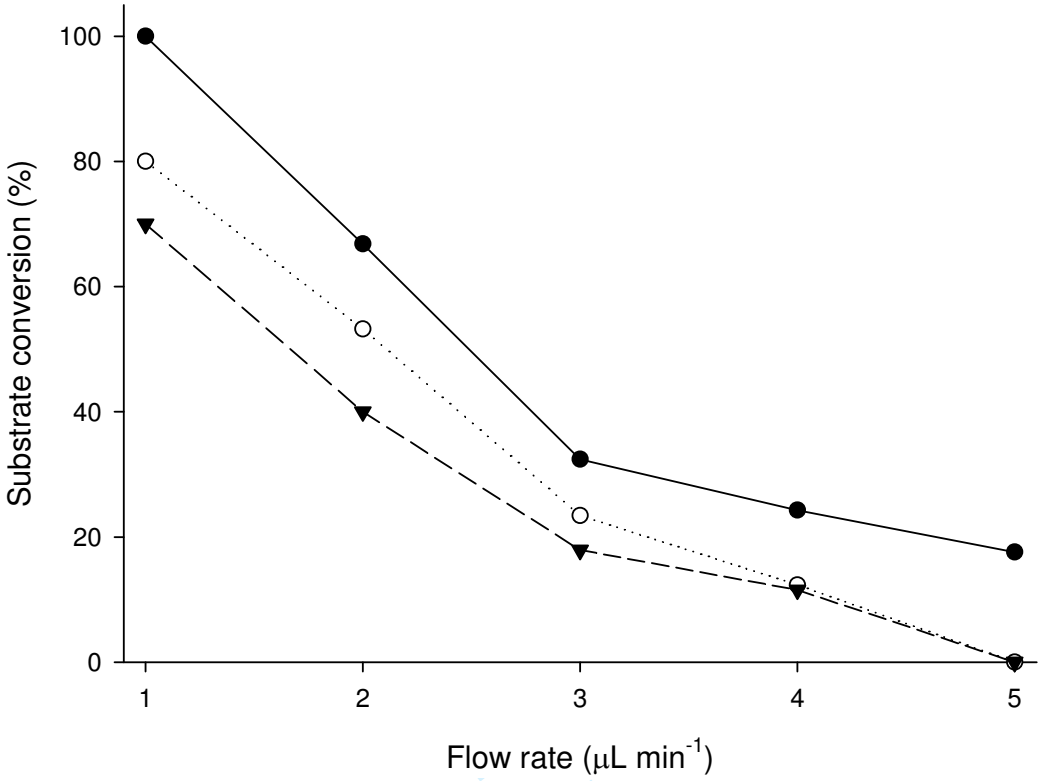
Substrate conversion (%)
100
80
60
40
20
0
1
2
3
4
5
Flow rate (μL min-1)

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