doi:10.2533/chimia.2010.799 Chimia 64 (2010) 799–802

Continuous Micro-Production Using Enzymatic Reaction and Online Monitoring

Regula Moser, Thierry Chappuis, Ennio Vanoli, Simon Crelier, and Olivier Naef*

Abstract: A micro-reactor coupled to a microfluidic system and an online UV/VIS spectrometer is described. The enzymatic reaction studied is the hydrolysis of the N-benzoyl-L-tyrosine ethyl ester (BTEE) to N-benzoyl-L-tyrosine (BT) and ethanol, catalyzed by chymotrypsin. The production is online monitored with UV spectroscopy at 256 nm. Three different immobilization methods of the enzyme are discussed: Eupergit® C, controlled-pore glass (CPG), and Sepharose.

Keywords: Chymotrypsin · Enzyme immobilization · Enzymatic reaction · Micro-production · Micro-reactor

Introduction

Nowadays the placement of new products on the market demands more and more flexibility from industry. The competition to innovate becomes tougher, especially in the domains of chemistry and biochemistry. In this context, reducing the development costs and optimizing production processes are valuable ways of gaining a competitive advantage.

The development time of a chemical or biochemical process can be reduced significantly by using a micro-reactor. Indeed, due to the low volumes involved, tests can be carried out with small amounts of reactants. Moreover, as micro-reactors have an advantageous surface-to-volume ratio, the temperature of the reaction mixture can be controlled more efficiently than in larger scale reactors. Hence, the safety of the process is better guaranteed and, due to the continuous nature of tubular micro-reaction systems, steady-state conditions can be achieved easily. Then, when the process is sufficiently mature, further scale-up and transfer to production can be envisaged by parallelization.[1]

The development of micro-reactors integrating enzymatic catalysis is a topic of current interest. The environment-friendly nature of enzymatic conversion is attractive in this context. Micro-reactors have been used to carry out enzymatic reactions both with the enzyme in solution or immobilized on a support. Immobilization is usually preferred, since the enzyme can be recycled and reused during the entire lifetime of its activity. Thus much research effort has been invested in this field. In the review published by Miyazaki and Maeda,[2] typical techniques for the preparation of immobilized-enzyme micro-channel reactors are listed. The most widespread techniques for enzyme immobilization use particle entrapment or immobilization on surfaces or membranes. Immobilization on surfaces or membranes is generally performed by covalent binding with a crosslinker or by adsorption.

In the present study, three different methods to immobilize α-chymotrypsin in a micro-reactor are presented. Chymotrypsin is a protease which is used, in this example, to hydrolyze an ester to a carboxylic acid. In industry, chymotrypsin is used for example to produce L-phenylalanine from the racemate mixing or peptide mapping. The supports used to achieve enzyme immobilization include Eupergit® C and Sepharose polymers and controlled pore glass (CPG). The chymotrypsin is always immobilized by covalent binding on the support.[3]

Materials and Methods

Chemicals

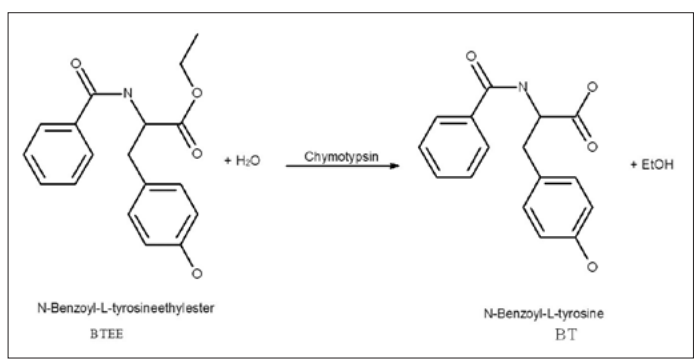
In order to perform the chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-tyrosine ethyl ester (BTEE) to N-benzoyl-L-tyrosine (BT) and ethanol (Scheme 1), α-chymotrypsin from bovine pancreas and N-benzoyl-L-tyrosine ethyl ester were purchased from Sigma-Aldrich and used without further purification.

For the immobilization of the α-chymotrypsin, Eupergit® C, Sepharose 6B were purchased from Sigma and Controlled Pore Glass (120–200 mesh and 200–400 mesh) from Fluka. These supports were used without further treatment prior to enzyme immobilization.

Methanol and hydrochloric acid were purchased from Merck, potassium dihydrogenphosphate was purchased from Riedel-de-Haën and disodiumhydrogenphosphate from Fluka. Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich. These chemicals were all used without further purification.

Homogeneously Catalyzed Hydrolysis Reaction

Before immobilization of the enzyme on different supports, the hydrolysis of the BTEE to BT and ethanol was first stud-



Scheme 1. Hydrolysis of N-benzoyl-L-tyrosine ethyl ester (BTEE) to N-benzoyl-L-tyrosine (BT) and ethanol.

*Correspondence: Prof. O. Naef
Ecole d'ingénieurs et d'architectes de Fribourg
Boulevard de Pérolles 80
CP 32
CH-1705 Fribourg
Tel.: +41 26 429 67 05
E-mail: olivier.naef@hefr.ch

ied in a batch setup in order to determine the evolution of the absorbance of a 0.56 mM BTEE solution in the reaction buffer as a function of time, with chymotrysine used in solution as a homogeneous catalyst. The reaction buffer is prepared as a 1.42:0.9:0.08:0.1 solution of Tris/HCl buffer at pH 7.8, methanol, calcium chloride and HCl. As a comparison, the evolution of the absorbance in a 0.56 mM BTEE solution in the reaction buffer in the absence of chymotrypsin was also monitored.

Immobilization on Eupergit® C

Eupergit® C is an acrylic polymer with porous structure vested by epoxy groups on the surface. The immobilization takes place by covalent binding with the epoxy groups.

The immobilization of α-chymotrypsin proceeded as described in the literature.[4,5] The immobilization was done in a phosphate buffer, 1 M at pH 7, room temperature, for 20 h. For the coupling reaction 1 g of Eupergit® C was reacted with 9 ml of enzyme solution (concentration between 1–5 mg/ml) in a reaction tube.

The analysis of the activity (SIGMA Quality control Test, Enzymatic Assay of chymotrypsin EC 3.4.21.1) and of the remaining protein concentration (BIO-RAD, Quick Start™ Bradford Protein Assay) in the reaction mixture permitted the control of the process. The difference between the concentration at the beginning and the concentration at the end can be considered as the concentration immobilized on Eupergit® C.

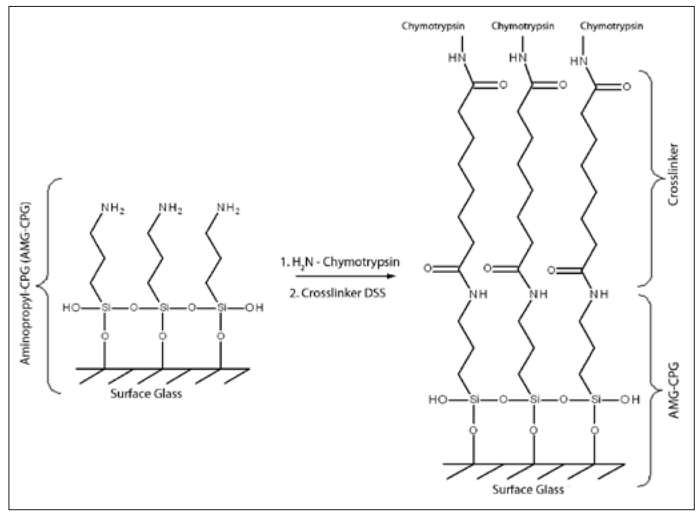
Immobilization on Sepharose® 6B

Sepharose® 6B is a polysaccharide with reactive epoxy groups, which are involved in the immobilization process of chymotrypsin the same way as described above for Eupergit® C supports. Sepharose® 6B particles have a size distribution of 45–165 μm.

About 1 g of Sepharose® 6B was rinsed with deionised water until it swelled up three times its dry volume. To start the reaction, the swelled Sepharose® 6B was transferred in a tube, and then 5 ml of the solution of chymotrypsin were added to the Sepharose® 6B. The dispersion was shaken for 23 h at room temperature.

After the immobilization of chymotrypsin the remaining amine groups were deactivated by rinsing first with water, afterwards 5 ml of ethanolamine 1 M were added and the dispersion was shaken overnight (23 h) at room temperature. To remove excess uncoupled ligands after coupling, the Sepharose® 6B was washed alternatively with high and low pH buffer solution at least three times. Starting with Tris/HCl buffer pH 8, following with acetate buffer pH 4. To store finally the Sepharose® with the immobilized chymotrypsin, 3 ml of HCl 0.1 mM and $CaCl_2$ 10 mM solution (300 μl HCl 1 mM, 60 μl $CaCl_2$ 2 M and 2.64 ml of water) were added. Once the treatment was finished, the product was stored in the fridge at 4 °C.

To follow the reaction, the activity and the protein concentration was analyzed as described above for Eupergit® C.



Scheme 2. Binding reaction between CGP particles and α-chymotrypsin using disuccinimidyl suberate (DSS) as a crosslinker.

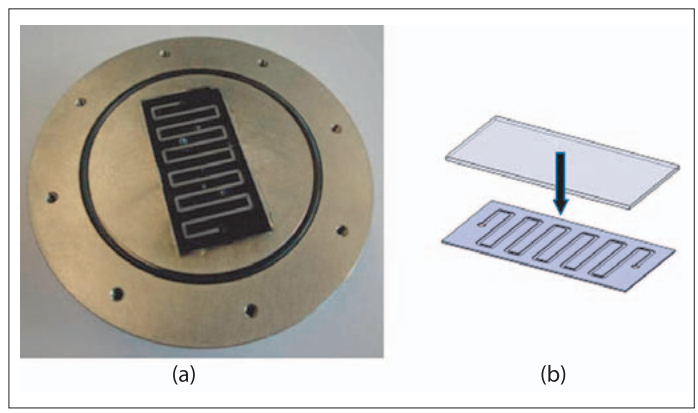


Fig. 1. (a) Micro-reactor and (b) reaction channel used to study the chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-tyrosine ethyl ester (BTEE) to N-benzoyl-L-tyrosine (BT) and ethanol.

Immobilization on Controlled Pore Glass (CPG)

Controlled Pore Glass (CPG) is a new packing material used for chromatography columns. It is composed of almost pure quartz glass. It is chemically inert and very hard. Activated CPG has a functionalized surface containing aminopropyl groups. Convalent binding between α-chymotrypsin and the CPG particles is achieved by using disuccinimidyl suberate (DSS) as a crosslinker. The binding reaction of α-chymotrypsin on CPG particles is illustrated in Scheme 2.

First of all, the CPG was swelled to about three times its dry volume with distilled water. Afterwards, the CPG was transferred to a vial and a solution of 2 mg/ml chymotrypsin in borax buffer pH 8 was added. This mixture was stirred for 15 min to form intermolecular bindings. This was followed by adding a solution of disuccinimidyl suberate (DSS) solution (3.6 mg DSS in 100 μl N,N-dimethylformamide). After stirring for 30 min, the reaction was complete. At the end, the CPG is rinsed with water, before storing in HCl 0.1 mM and $CaCl_2$ 10 mM solution at 4 °C.

Backed-bed Reactor and Micro-reactor Systems

After immobilization of the enzyme on different types of supports, the immobilized enzyme was filled as a packed-bed either in a tube (glass or stainless steel, internal diameters varying between 0.5 and 5 mm) or in a micro-reactor. Eupergit® C and Sepharose® 6B carriers were only tested in tubes while CPG-enzyme catalysis was also tested in the micro-reactor.

The tubular micro-reactor used to study the chymotrypsin-catalyzed reaction presented here is represented on Fig. 1a. This micro-reactor was manufactured at University of Applied Sciences Western Switzerland (HES-SO) La Chaux-de-Fonds (HE Arc, Switzerland) and further tested and characterized at HES-SO Fribourg (EIA-FR, Switzerland). The channel was produced from a silicon wafer etched by a laser. Its dimensions are 0.3 mm in width and 1 mm in height. Holes were positioned at each end of the channel to allow both inflow and outflow of the liquid and the whole channel structure was covered by a glass plate fixed to the silicone structure by anodic bonding (Fig. 1b). The micro-

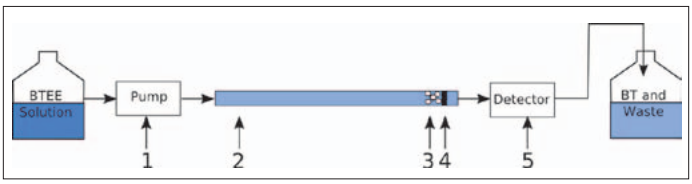


Fig. 2. Schematic representation of the process flow. 1 is the pump, 2 is channel, 3 enzyme support, 4 is a filter and 5 is the detector.

Table 1. Overview of the process flow

1	2	3	4	5
Pump	**Channel**	**Enzyme support**	**Filter**	**Detector**
Peristaltic pump	Glass tube	CPG 120–200 mesh Eupergit® C	Fibreglass	UV/VIS spectrometer with flow-through cell
HPLC pump	Stainless steel tube	CPG 120–200 mesh und 200–400 mesh Eupergit® C	HPLC column filter, Poren Ø 0.5 μm	HPCL Spectrometer UV/VIS
HPLC pump	Micro-reactor	CPG 200–400 mesh	Fibreglass	HPCL spectrometer UV/VIS

structured channel was then sandwiched in a housing made of stainless steel.

The liquid flow across the system was forced by an HPLC pump and a UV/VIS detector was positioned at the outflow end of the micro-reactor. This setup was used to analyze the hydrolysis of N-benzoyl-L-tyrosine ethyl ester (BTEE) to N-benzoyl-L-tyrosine (BT) and ethanol (Fig. 2). The reaction was analyzed at 256 nm. Fig. 2 and Table 1 show a conceptual overview of the experimental setup. As an enzyme, α-chymotrypsin from bovine pancreas was chosen. The advantages of α-chymotrypsin are a good thermal- and activity stability (storage at 4 °C allows the conservation of the activity during a maximum of 2 months) and quick reactivity at 20 °C.

Results and Discussion

Immobilization of the Enzyme

Chymotrypsin was immobilized on three different supports to compare the activity of the immobilized enzyme with continuous flow hydrolysis of BTEE to form BT and ethanol. In order to monitor the immobilization reaction, the liquid supernatant was analyzed to determine the remaining activity of enzyme (Sigma Quality Control Test Procedure, Enzymatic Assay of chymotrypsin, EC 3.4.21.1) and the protein concentration (Quick Start Bradford Protein Assay, Instruction manual, Bio-Rad) in the solution. These measures for the immobilization of chymotrypsin on Eupergit® C are presented as a function of time on Fig. 3. As seen in Fig. 3, enzyme activity and concentration in the liquid supernatant evolve in parallel. Thanks to the results, the yield of the immobilization reaction could be determined. In addition to the yield, the stability of the chymotrypsin could be determined by calculating the ratio of the activity to the total protein concentration. If the enzyme is not degraded, this ratio should reach a stable value during the reaction. The amount of chymotrypsin immobilized on the support and the yield of the immobilization reaction is reported in Table 2 for Eupergit® C, Sepharose® 6B and Controlled Pore Glass.

As reported in Table 2, it was possible to immobilize chymotrypsin on Sepharose, but the amount of enzyme immobilized per gram of Sepharose is very low with a poor yield of the immobilization reaction. According to these results, the technique of immobilizing chymotrypsin on Sepharose® 6B carriers was not carried forward for tests with the hydrolysis reaction in continuous flow tubes.

The amount of α-chymotrypsin immobilized on Eupergit® C proved to be larger than on Sepharose® 6B and Controlled Pore Glass. While testing the hydrolysis reaction in a continuous flow packed bed tube, a conversion of 90% was achieved after 0.3 min. of contact with the immobilized enzyme.

In spite of a larger immobilization yield, the amount of α-chymotrypsin immobilized on Controlled Pore Glass is about 30% lower than that measured for Eupergit® C. During the hydrolysis reaction, chymotrypsin immobilized on Controlled Pore Glass enabled a 95% conversion of BTEE to BT when the continuous tubular reactor was packed with a bed length of 2.4 cm.

Fig. 4 shows the comparison of the BT concentration profiles measured during the hydrolysis reaction for different contacting times between the liquid flow and the immobilized phase. Here, the reaction tube is packed with varying length beds of both Eupergit® C and CPG. The results demonstrate good stability of the chymotrypsin during the experiments. Moreover, the chymotrypsin immobilized on CPG is clearly more active than the enzyme immobilized on the Eupergit® C support.

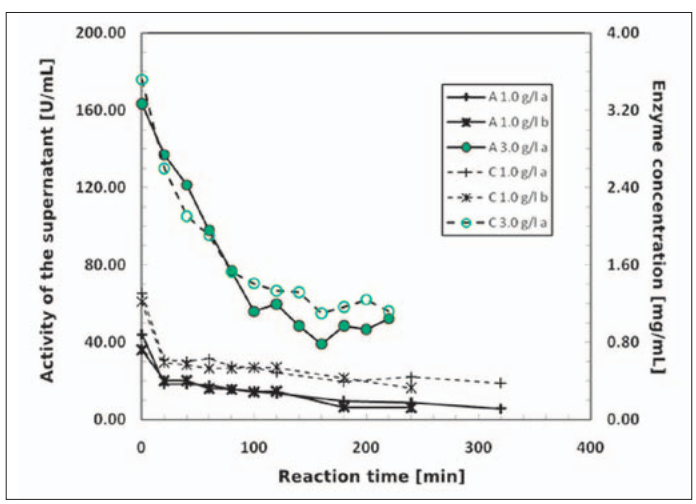


Fig. 3. Activity and enzyme concentration of the supernatant liquid during chymotrypsin immobilization on Eupergit® C.

Table 2. Amount of α-chymotrypsin immobilized on each support and yield of the immobilization reaction.

Support	Chymotrypsin on support [mg/g]	Yield of the immobilization reaction [%]
Sepharose	4.6	46
Controlled Pore Glass	9.3	94
Eupergit C	13.5	50

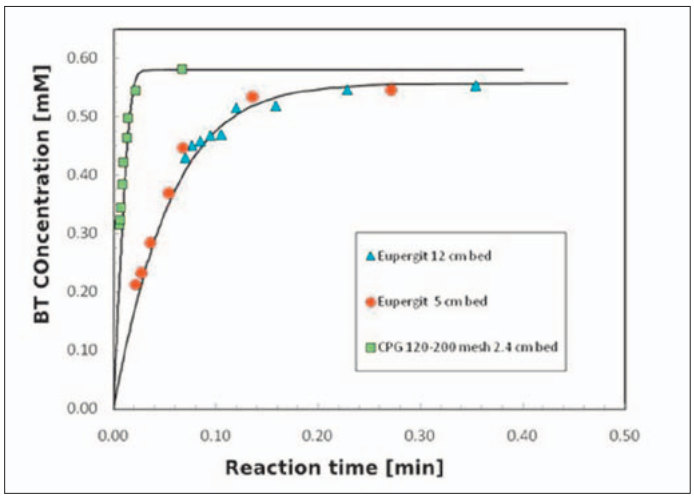


Fig. 4. BT concentration profiles for the hydrolysis reaction carried out in a continuous tube packed with varying length beds of Eupergit® C and Controlled Pore Glass 120–200 mesh at 20 °C and pH 7.8.

This can be seen from the much larger rate of formation of BT with Controlled Pore Glass.

Fig. 4 also shows that the length of the Eupergit® C with chymotrypsin has almost no influence on the kinetics of the kinetics of BTEE to BT.

Figs 5 and 6 depict the conversion and the productivity of the BTEE to BT hydrolysis reaction implemented in a micro-reactor using enzyme immobilization on 120–200 mesh Controlled Pore Glass. Fig. 5 shows a slight decrease of the conversion as the flow rate of the reaction mixture increases. However, the conversion remains over 80% for most tests. As a consequence, immobilization of chymotrypsin in the micro-reactor allowed a linear increase of productivity as a function of the linear velocity of the liquid phase.

Optimization still has to be performed with the micro-reactor in order to maximize the productivity in a micro-production system such as the micro-channel device described here. In this study, the reaction and the enzyme were chosen as a model able to demonstrate the feasibility and the potential advantages of immobilizing an enzyme on a solid support directly packed in the reactor. As seen with Eupergit® C and Controlled Pore Glass (CPG), a larger amount of enzyme immobilized on Eupergit® C did not necessarily result in a higher activity of the immobilized system. Indeed, the activity of an enzyme also depends on its binding site. If the active site is distant enough from the binding site, the enzyme is likely to retain its activity. However, if the distance between these sites is too short, the binding of the enzyme to the support could alter the activity of the catalyzing site. Hence, the method used for enzyme immobilization in a micro-reactor is both support- and enzyme-dependent. It is probably the most limiting step for such a micro-reactor to be of widespread use for real-life chemical and biochemical developments involving enzyme catalysis. Once the immobilization step is mastered with maximized activity of the active substance, further development with the micro-reactor proved to be very flexible, allowing steady production with high conversion on a continuous basis. Solutions for fast and low-cost design and fabrication of customized microchannel-based reactors are currently in development and have the potential to pave the way for faster process development and reduced scale-up and optimization costs.

Conclusion

In the present article, the realization of the enzyme-catalyzed hydrolysis of BTEE to BT and ethanol in a continuous flow micro-reactor has been presented. Immobilizing the enzyme on a solid support clearly appeared to be the limiting step, since it was proved to be both support- and enzyme-dependent. In particular, Eupergit® C was able to retain more chymotrypsin but with a reduced activity compared to Controlled Pore Glass (CPG). Hence only the latter immobilization technique was used to pack the micro-reactor with the catalyzing substance. Micro-production experiments demonstrated interesting conversions at steady-state and stable productivity up to more than 18 mg/h of BT.

Acknowledgements

The project is funded by the University of Applied Sciences of Western Switzerland (HES–SO) through the HES–SO CALL 2007.

Received: September 6, 2010

[1] K. Geyer, J. D. C. Codée, P. H. Seeberger, *Chem. Eur. J.* **2006**, *12*, 8434.
[2] M. Miyazaki, H. Maeda, *TRENDS Biotechnol.* **2006**, *24*, 463.
[3] R. Nicoli, S. Rudaz, C. Stella, J.-L. Veuthey, *J. Chromatogr. A* **2009**, *1216*, 2695.
[4] C. Mateo, O. Abian, R. Fernandez–Lafuente, J. M. Guisan, *Enzyme Microb. Technol.* **2000**, *26*, 509.
[5] M. J. Hernaiz, H. G. Crout, *Enzyme Microb. Technol.* **2000**, *27*, 26.

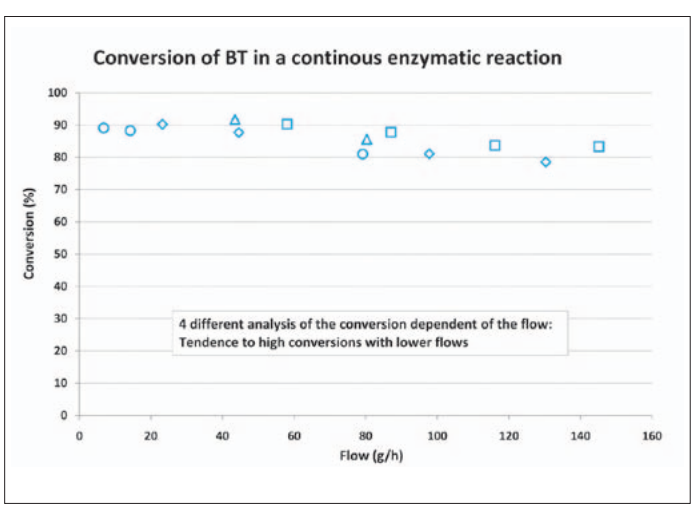


Fig. 5. Conversion of BTEE to BT as a function of the rate of the flowing phase through a continuous micro-reactor packed Controlled Pore Glass.

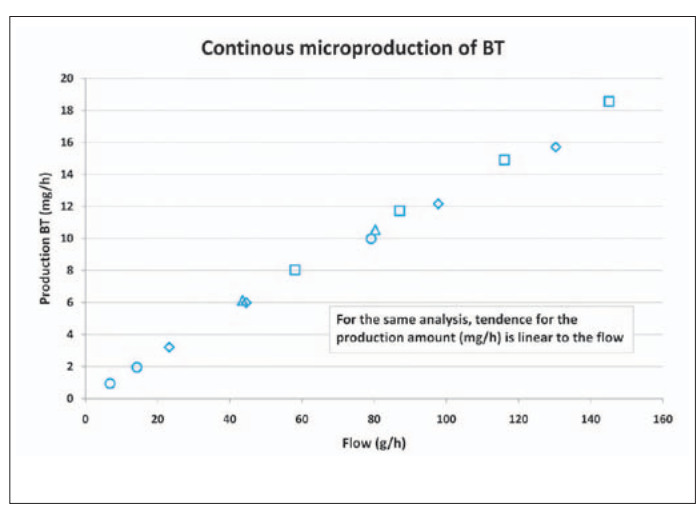


Fig. 6. Productivity of a continuous flow micro-reactor with packed Controlled Pore Glass for the chymotrypsin-catalyzed hydrolysis of BTEE to BT.