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Cephalexin synthesis by immobilised penicillin G acylase under non-isothermal conditions: reduction of diffusion limitation

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Abstract

The effect of thermodialysis on the enzymatic kinetic synthesis of the antibiotic cephalexin was investigated. As reference points, two existing models for an immobilised enzyme (Assemblase®) and for the free enzyme were used. For Assemblase®, it is known that diffusion limitation occurs and that therefore considerably more of the undesired side-product phenylglycine is formed.

The enzyme was immobilised on a membrane, and under isothermal conditions (293 K) the course of the reaction resembled that of the Assemblase® enzyme. However, if a temperature gradient was applied across the membrane, with an average temperature of 293 K for the enzyme, than the course of the reaction changed. For large temperature gradients (30° and more), the course of the reaction resembled that of free enzyme. Thermodialysis enhances mass transfer across the membrane and therewith reduces diffusion limitations in the immobilised enzyme on the membrane.

The stability of the immobilised enzyme is such that the reactor can be re-used repeatedly. This, together with the positive effect of the temperature gradient on the course of the reaction, makes thermodialysis an interesting new technique that has potential to be applied on a larger scale if the membrane surface area per volume of reactor can be improved.

Keywords: Thermodialysis reactor; Cephalexin; Penicillin G acylase; Immobilisation; Grafted membranes

1. Introduction

One of the most important reasons to immobilise an enzyme is the possibility of re-use. The product can (relatively) easily be separated from the enzyme and continuous operation may become an option. However, there are also some drawbacks connected to

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immobilisation such as a loss of activity upon immobilisation, which preferably should be compensated by increased enzyme stability. Further, diffusion limitation may occur which reduces the productivity of the immobilised enzyme (e.g. [1]).

One of the few techniques that can be used to overcome diffusion limitation is thermodialysis. For thermodialysis, the enzyme has to be immobilised onto a membrane and a temperature gradient has to be applied over this membrane. Under these conditions, a volume flow of liquid is observed together with differential solute fluxes. A comprehensive overview of the fundamentals of this technique and the applications that were studied is given by Mita [2]. Up till now, thermodialysis has been used for hydrolysis reactions. In case of *hydrolysis* of cephalexin, an increase in reaction rate was found with increasing temperature gradient indicating that diffusion of cephalexin was enhanced by the applied gradient [3].

In previous research, we studied enzymatic antibiotic *synthesis* carried out both with free and immobilised enzyme (Assemblase®) using cephalexin as a model component and found that diffusion limitation occurs in Assemblase® [4,5]. Cephalexin, a cephalosporin-type of antibiotic can be produced via a kinetic reaction using activated phenylglycine and 7-aminodeacetoxylcephalosporinic acid (7-ADCA) as substrates [6]. As an activator-group both esters and amides can be used. Besides synthesis of the antibiotic, also enzyme-catalysed hydrolysis of the activated phenylglycine and of the cephalexin takes place [7,8].

In this study, cephalexin synthesis is investigated which implies that not only diffusion of cephalexin should be influenced but also diffusion of 7-ADCA and activated phenylglycine should be influenced accordingly. We investigated the influence of the temperature gradient on the course of the cephalexin synthesis reaction. The results were compared to the model predictions for free enzyme and immobilised Assemblase®. The reaction conditions were investigated and the difference between the measured course of the reaction and the model prediction for free enzyme (situation without diffusion limitation) was monitored. Further, the stability of the enzyme was investigated; and process concepts based on the thermodialysis reactor are discussed.

2. Materials and methods

2.1. Chemicals

The water used throughout was double distilled. Phenylglycine amide and 7-ADCA, were in-house chemicals of DSM Research (Geleen, The Netherlands) and DSM Anti-Infectives (Delft, The Netherlands). Sodium hydroxide (>99%) and hydrochloric acid were from Merck (Darmstadt, Germany). All other chemicals were from Sigma-Aldrich (Dorset, UK) unless stated otherwise.

2.2. Enzyme

Penicillin G acylase from *E. coli* is an in-house enzyme of DSM Anti-Infectives (Delft, The Netherlands). The enzyme was isolated as described by Kaasgaard et al. [9]. The amoxicillin synthesising activity of the enzyme was 1360 units/ml (one unit is the amount of enzyme that produces 1 g of amoxicillin·$3H_2O$ from 6.5% 6-amino-penicillic acid and 6.5% hydroxyphenylglycine methyl ester per minute at pH 6.7 and 293 K). The enzyme was stored at 4°C.

2.3. Cephalexin synthesis

Phenylglycine amide and 7-ADCA are dissolved in water in a concentration of 100 mM. The reaction mixture (50 ml) is brought to the desired temperature and pH (starting pH is 8 in all cases). After the temperature has become constant, circulation through the membrane module is started. During the first hour, every 15 min a sample is taken. After that, every 30 min a sample is taken till a total reaction time of 4 h is reached. For all experiments, the pH is checked at the end of the experiment and it was found that it never exceeded 8.5. From previous research, it is known that the course of the reaction is not different for a pH between 7.5 and 8.5, therefore the models described in the next paragraph can be used to analyse the course of the reaction [4].

2.4. Kinetic model

In Fig. 1, a schematic representation of the kinetic model is shown. It is based on the reaction mechanism of penicillin G acylase derived by Duggleby et al [10].

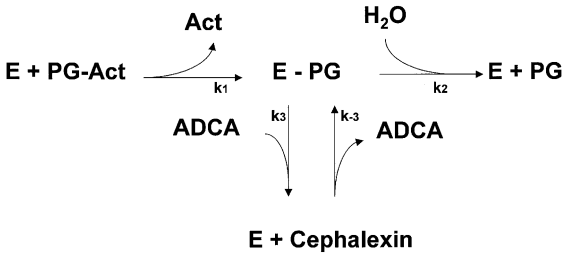

Fig. 1. Simplified model for kinetic cephalexin synthesis (adapted from [4]). PG-act denotes activated phenylglycine, E is free enzyme, E-PG is enzyme phenylglycine complex, ADCA is 7-aminodeacetoxycephalosporinic acid, PG is phenylglycine and CEX is cephalexin.

First, the activated phenylglycine binds to the free enzyme giving an enzyme phenylglycine complex. This complex can either be hydrolysed by water to give free enzyme and phenylglycine or can react with 7-ADCA to give cephalexin and free enzyme. The cephalexin can in turn bind to the free enzyme to give the enzyme phenylglycine complex back [4].

For the model shown in Fig. 1, reaction rate equations were derived using the King–Altman method (Eq. (1)–(4)). In addition, mass balances for activated phenylglycine and 7-ADCA were used.

$$\frac{\mathrm{d[CEX]}}{\mathrm{d}t} = \frac{k_1 k_2' [\mathrm{ADCA}][\mathrm{PGA}] + k_2' k_{-3} [\mathrm{CEX}]}{\Sigma} E_0 \quad (1)$$

$$\frac{\mathrm{d[PG]}}{\mathrm{d}t} = \frac{k_1 k_2' [\mathrm{PGA}] + k_2' k_{-3} [\mathrm{CEX}]}{\Sigma} E_0 \quad (2)$$

$$\frac{\mathrm{d[PGA]}}{\mathrm{d}t} = \frac{-k_1 k_2' [\mathrm{PGA}] + k_1 k_{-3} [\mathrm{PGA}][\mathrm{ADCA}]}{\Sigma} E_0 \quad (3)$$

$$\frac{\mathrm{d[ADCA]}}{\mathrm{d}t} = \frac{\mathrm{d[CEX]}}{\mathrm{d}t} \quad (4)$$

$$\Sigma = k_1 [\mathrm{PGA}] + k_2' + k_3 [\mathrm{ADCA}] + k_{-3} [\mathrm{CEX}] \quad (5)$$

All concentrations are in mmol per kg total; the enzyme fraction, E_0, is in grams of enzyme per gram total. The constants (k_1, k_2, k_3 and k_{-3}) were calculated for both free enzyme and immobilised enzyme (Assemblase®). The values of these constants at 293 K are given in Table 1. For a more detailed description, we would like to point to [5].

Table 1
Kinetic constants for free enzyme and the apparent kinetic constants of Assemblase® at 293 K and pH 8[a]

Constant	Free enzyme	Assemblase®
k_1 (1/min)	17.3	6.1
k'_2 (mmol/l/min)	741	127
k_3 (1/min)	172	4.0
k_{-3} (1/min)	111	5.2

[a] The enzyme content in the free enzyme is considerably higher than in the Assemblase® and that results in the higher 'constants' for free enzyme.

Two mass balance equations, one for the nucleus (ADCA) and one for the side-chain (phenylglycine), are valid. In case no product is present at time zero, the mass balance equations reduce to Eqs. (6) and (7), in which the subscript 0 denotes the concentration at time zero.

$$[\mathrm{ADCA_0}] = [\mathrm{ADCA}] + [\mathrm{CEX}] \quad (6)$$

$$[\mathrm{PGA_0}] = [\mathrm{PGA}] + [\mathrm{PG}] + [\mathrm{CEX}] \quad (7)$$

For Assemblase® and free enzyme, the course of the reaction could be described accurately. If both enzymes were compared than it became clear that at 293 K the immobilised Assemblase® produces significantly higher amounts of the by-product phenylglycine and slightly lower amounts of cephalexin [5]. It was shown previously that this is caused by diffusion limitation of both the synthesis reaction [5] and of the cephalexin hydrolysis reaction [3].

2.5. Comparison of model with measured concentrations

The model is used to compare the experimental results of the thermodialysis reactor with those obtained for batch reactions carried out with free enzyme or Assemblase®. Because the enzyme concentration on the membrane could not be measured, the amount of enzyme in the model (E_0) is chosen in such a way that the cephalexin concentration can be described well. The nucleus mass balance (Eq. (6)) dictates that in that case also the ADCA concentration is predicted correctly. The difference between the model and the measured concentrations is only reflected in the phenylglycine and the phenylglycine amide

concentration. A positive difference for one of these components with the model is reflected as an equivalent negative difference for the other component, because of the other mass balance (Eq. (7)). This makes it possible to discuss the effects of diffusion limitation using only one of the components. In this paper, we opted for phenylglycine.

The model lines that are shown in this paper are all constructed with the free enzyme model with the exception of Fig. 3a.

2.6. Membrane modification and enzyme immobilisation

Nylon hydrolon membranes, from the Italian Division of Pall (Pall Italia srl, Milan, Italy) were used as carriers for the enzyme. The nominal pore size is 0.2 μm and the membrane surface area in the module is approximately 45 cm^2.

Before enzyme immobilisation could take place, the membranes were first grafted and subsequently activated. For grafting, the membranes were immersed in a 1:1 (v/v) ethanol in water solution containing 10% (v/v) methylmethacrylate. Subsequently, they were treated by γ-ray irradiation for 8 h (Cesium 137 source in a 1000 Elite gammacell from Nordion International Inc. Canada). The average dose in the core of the radiation chamber was 2.35×10^4 rad/h. After this, the membranes were repeatedly washed with water to remove the unbound methylmethacrylate. The membranes were dried; for the experimental conditions used in this paper the grafting-percentage is 43% (g/g untreated membrane). After the grafting procedure, the membranes were soaked in a 10% (v/v) hexamethylenediamine aqueous solution for 1 h at room temperature. After this, the membranes were first repeatedly washed with water and subsequently immersed for an hour in a 2.5% (v/v) glutaraldehyde aqueous solution. After that, the membrane was washed again with water. Onto the thus activated membranes, the enzyme was immobilised. At 277 K, the membranes were immersed for 20 h in a 0.1 M phosphate buffer (pH 7.0) containing 15% (v/v) of the original enzyme solution. After repeated rinsing with water, the membranes were ready for use in an experiment. Between experiments the membranes were stored in a 0.1 M phosphate buffer (pH 7.0) in a fridge. More details on the enzyme-immobilisation procedure are given in one of our previous publications on cephalexin hydrolysis [3].

2.7. Thermodialysis reactor

In Fig. 2, a schematic representation of the thermodialysis reactor is given. The membrane containing the immobilised enzyme is mounted in the membrane

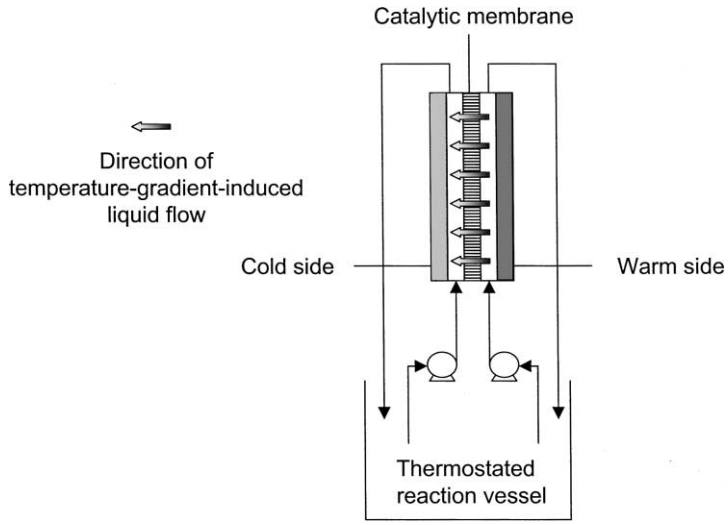

Fig. 2. Schematic representation of the thermodialysis reactor.

module. The reaction mixture is continuously recirculated through the module. The liquid flow through the tubing is approximately 100 ml/h, both pumps working at the same flow. In previous research, the influence of the pumps on diffusion was investigated and it was found that this effect could be excluded (e.g. [2]). The internal volume of the module and the tubing is small (<10 ml) and therefore the residence time in the module is relatively short. It can safely be assumed that the concentration in the module is not different from the concentration in the stirred vessel.

The membrane module consists of two half cells that both are connected to water-baths that control the temperature in the membrane chambers. The thermodialysis reactor is operated under isothermal and non-isothermal conditions. If a temperature difference is applied, a liquid flow from the warm side to the cold side is initiated resulting in increased mass transfer or stated differently, in a situation in which diffusion limitation is less likely to occur. An extensive description of the phenomena occurring (and the thermodialysis reactor) is given in the review-paper of Prof. Mita [2]. Please note that the temperature is at its most extreme at the outside of the membrane module. The actual temperature difference over the membrane is considerably smaller than the temperature gradient across the module. In one of our previous publications, we reported that the temperature difference across the membrane is approximately 2° for a temperature difference of 20° across the module [2,3]. The temperature that the enzyme in the centre of the module is exposed to is approximately equal to the average temperature.

2.8. Analysis

2.8.1. Sampling and sample preparation

Samples of 0.25 ml were taken from the reaction mixture and put into a measuring flask. Distilled water was added until a known volume was reached and the contents were mixed. A 1.0 ml sample was taken from this mixture and centrifuged at 278 K during 15 min at 15.300 rpm (Beckman GS-15R, rotor: Beckman F2402H). After centrifugation, a 0.75 ml sample was taken and analysed by HPLC.

2.8.2. HPLC

The HPLC system was purchased from Thermo Separation Products (TSP, Breda, The Netherlands) and consisted of a SCM 1000 vacuum membrane de-gasser, a SpectraSystem P4000 gradient pump, a SpectraSystem AS autosampler and a SpectraSystem UV3000 detector.

The columns were of type reversed phase C18 and were purchased from Bester (Amstelveen, The Netherlands). The pre-column was of type SGE W5C18RS, (10 mm × 4 mm). The main column was of type Prodigy ODS(3) (250 mm × 4.6 mm). The particle size was 5 μm and the pore size 10 nm.

The temperature of the column was 313 K and the tray temperature 278 K. The injected volume was between 1 and 20 μl. For elution, acetonitrile and 18.2 mM H_3PO_4 (pH 4.9) were used. The flow rate was 1.25 ml/min. Analysis took place both at wave lengths 191 and 265 nm. The retention times of the components were 3.3 min for phenylglycine (191 nm), 4.1 min for ADCA (265 nm), 5.0 min for phenylglycine amide (191 nm) and 9.9 min for cephalexin (265 nm). The concentrations were calculated using calibration curves. More details on the analysis method are given in [4].

3. Results and discussion

3.1. Isothermal cephalexin synthesis

Cephalexin synthesis was carried out under isothermal conditions at 293 K and the result was compared to the model predictions for immobilised Assemblase® and free enzyme. Because the actual amount of enzyme that was immobilised is not known, the model is fitted to the cephalexin concentration by adjustment of the amount of enzyme in the model as described in the Section 2. The result is shown in Fig. 3.

From Fig. 3 it becomes clear that the model for immobilised Assemblase® gives the best prediction of the course of the reaction. Both the cephalexin concentration and the phenylglycine concentration are very near to the model predictions. The experiment was repeated three times and in all cases, the same result was obtained; the immobilised Assemblase® model predicts the course of the reaction accurately. This indicates that also in this case diffusion limitation occurs. Another explanation is that the kinetics of the enzyme changed upon immobilisation. This

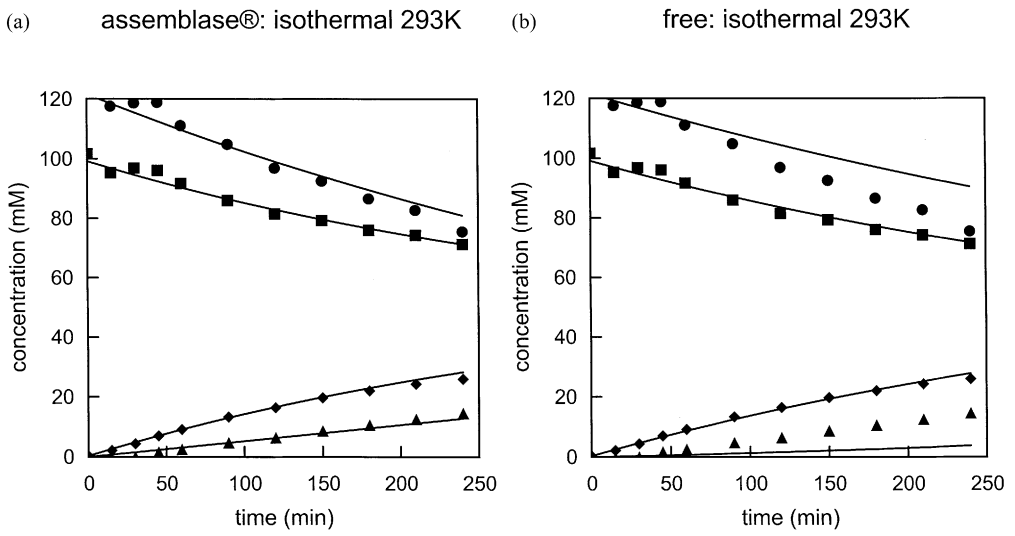

Fig. 3. Comparison of the models for free enzyme and immobilised Assemblase® for prediction of the course of the reaction during kinetic cephalexin synthesis under isothermal conditions (293 K). The symbols are (◆) cephalexin, (▲) phenylglycine, (●) phenylglycine amide and (■) 7-ADCA.

was investigated further by applying a temperature gradient over the membrane.

The result in Fig. 3b (measurement under isothermal conditions) shows, that differences between the model and measured concentrations are reflected in the phenylglycine- and phenylglycine amide concentration. Because a negative difference for e.g. phenylglycine corresponds to an equivalent positive difference for phenylglycine, the discussion will focus on phenylglycine only (see methods for an explanation of this choice). The discussion will also focus on the free-enzyme-model because it represents the ideal situation in which the reaction can be carried out free of diffusion limitation.

3.2. Non-isothermal synthesis at 293 K: dependence on temperature gradient

If the enzyme is limited by diffusion, than the course of the reaction can be influenced by application of a temperature gradient. The temperature gradient enhances mass transfer and therefore it is expected that the course of the reaction will start to resemble that of the free enzyme more with increasing temperature gradient. In Fig. 4, the temperature gradient is 10, 20, 30 and 38°. The average temperature, i.e. the temperature that the enzyme is exposed to, is always 293 K.

When comparing the model prediction with the measured concentrations for a temperature gradient of 10° than it becomes clear that the measured phenylglycine concentration is still considerably higher than predicted by the model for the free enzyme. If the temperature gradient is increased further than the difference becomes smaller. At the highest temperature gradient that was investigated, 38°, the difference is <4 mM after 240 min of reaction while the difference is >11 mM for a comparable experiment carried out under isothermal conditions. The difference in phenylglycine concentration for a temperature gradient of 30° is similar to that found for a temperature difference of 38°, therefore, increasing the temperature difference to >30° is not necessary. Clearly, the temperature gradient has a positive effect on the course of the reaction since the production of the unwanted side-product, phenylglycine, is suppressed.

3.3. Non-isothermal synthesis at temperature difference of 20°: dependence on average temperature

The effect of the temperature gradient on the course of the reaction is also investigated for different

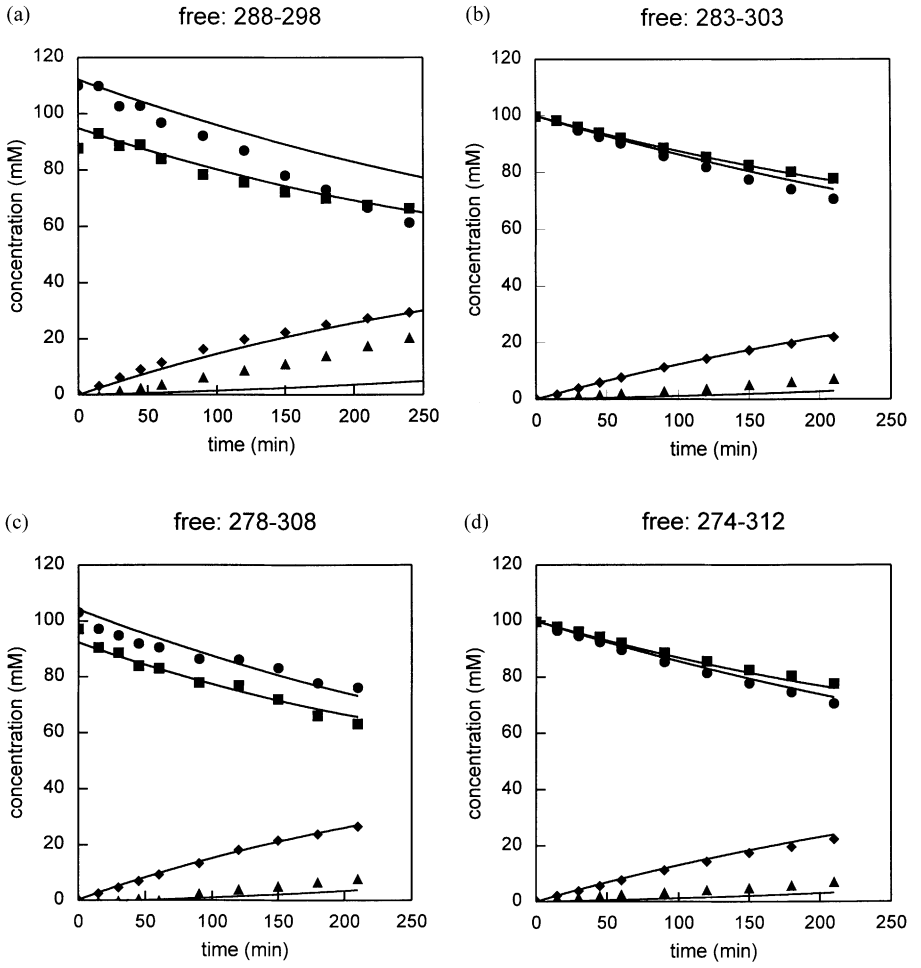

Fig. 4. (a–d) Prediction of the course of the reaction with the model for free enzyme. The temperature difference is 10, 20, 30 and 38° for the graph on the top left corner, the top right corner, the bottom left corner and the bottom right corner, respectively. The symbols are (◆) cephalexin, (▲) phenylglycine, (●) phenylglycine amide and (■) 7-ADCA.

temperatures, but now the average temperature is varied at a constant temperature gradient of 20°. The results are shown in Fig. 5.

As expected, at a high average temperature (293 and 298 K, Fig. 5c and d) the difference between model and measured concentrations is relatively big (compared to lower average temperatures). This is caused by diffusion limitation. At high temperature, both the reaction rate of the enzyme and the diffusion rate are higher. However, the reaction rate has a higher activation enthalpy than the diffusion rate, which means that the reaction is more temperature-dependent than the diffusion rate. Therefore, at high temperatures, the reaction will be more influenced by diffusion limitation. In this case, this implies that more phenylglycine will be formed. The same effect was previously found for reactions carried out with the immobilised Assemblase® [4].

With decreasing average temperature, the difference between the model and the measured concentrations becomes less. The explanation for this effect is the same as described before, diffusion limitation. At decreasing average temperatures, the diffusion rate will increasingly be able to keep up with the reaction rate and therefore the difference between the model and the measured concentrations will decrease.

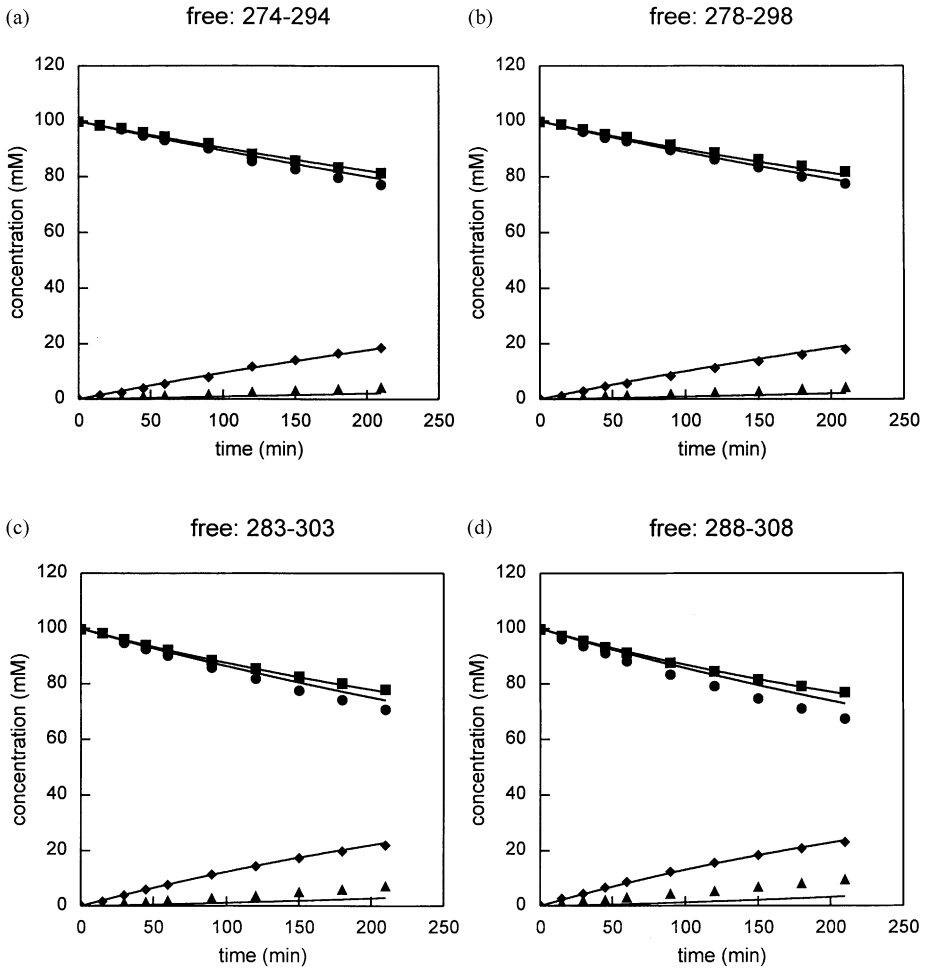

Fig. 5. (a–d) Prediction of the course of the reaction with the model for free enzyme. The temperature difference is in all cases 20°, the average temperature is 11, 15, 20 and 25° for the graph on the top left corner, the top right corner, the bottom left corner and the bottom right corner, respectively. The symbols are (◆) cephalexin, (▲) phenylglycine, (●) phenylglycine amide and (■) 7-ADCA.

For an average temperature of 283 K the difference between the model and the measured concentrations is <2 mM. This indicates that the performance of the thermodialysis reactor is very similar to that expected for a reactor based on free enzyme. However, in the case of the thermodialysis the product and enzyme can be separated easily while for free enzyme this separation may be more complicated.

3.4. Enzyme stability

For long term application of the thermodialysis reactor, it is important that the enzyme is sufficiently stable. All the experiments (11 in total) were carried out with the same membrane. In between measurements, the membranes were stored in the fridge. The total lifetime of the membrane was more than 1 month; the time that the membrane was used in the set-up was >50 h. In Fig. 6, the activity of the membrane is shown together with the temperatures that were used in the experiments. The experiments are shown in chronological order.

The activity of the membrane drops to approximately 50% of its original value after the first experiment, but stabilises after that. The values that we found are all between 43 and 60%. Because the reaction

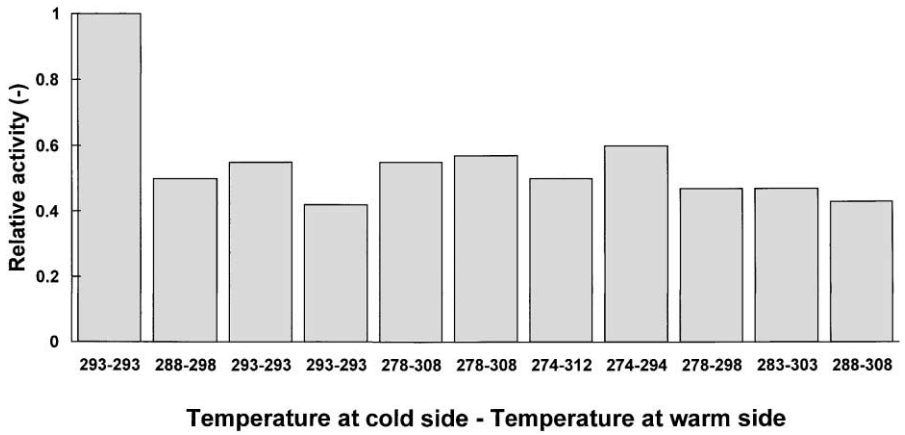

Fig. 6. The activity of the immobilised enzyme relative to the activity in the first experiment. The temperatures at the bottom indicate the temperatures at the warm and cold side of the membrane.

conditions varied between experiments, it is not possible to give an inactivation constant. However, based on the data in Fig. 6 it can be concluded that the immobilisation method that we used resulted in a stable enzyme that could repeatedly be used without too much loss of activity compared to the situation when it was used for the second time. We investigated whether free enzyme was released in the reaction mixture during the first experiment but this was not the case. We do not have an explanation for this initial loss of activity.

3.5. Processes based on thermodialysis: what needs to be done to make this possible?

The results shown up till now are very promising. The course of the synthesis reaction resembles that of the free enzyme when the average temperature is low. Further, the stability of the enzyme is such that repeated use is possible. These properties may make thermodialysis an interesting alternative for the current process based on immobilised Assemblase® [11,12].

On the other hand, there are also some drawbacks. The antibiotic and 7-ADCA are susceptible to chemical degradation [13,14]. And although the contact time with the warm side of the module is short, it is not advisable to use a temperature over 303 K in one side of the current module. Further, the activity per m^3 of reactor is relatively low. This 'problem' can be overcome if a higher enzyme loading, corresponding to more membrane surface area, can be used per m^3 of reactor. This implies the use of a different type of membrane like, e.g. hollow fibre membranes; this is currently investigated in one of our research groups (Prof. Mita). If a hollow fibre module can be used than also higher temperatures may become a possibility because the residence time in the thermodialysis reactor will be considerably shortened. In that case, the activity of the reactor will be high and diffusion limitation will be minimised.

4. Conclusions

The influence of thermodialysis on the course of the reaction during enzymatic cephalexin synthesis was investigated and we found that in all cases application of a temperature gradient across the membrane resulted in less by-product formation and therewith in a favourable situation compared to isothermal synthesis. The temperature gradient enhances mass transfer across the membrane and therewith diffusion limitations are (partly) lifted.

The immobilised enzyme could be used repeatedly. With the exception of the first run, the activity of the membrane was stable. This together with the fact that the course of the reaction can be influenced in a positive way by application of the temperature gradient makes thermodialysis an interesting new technique that also has potential for application on a larger

scale. Prerequisite is that the membrane surface area per volume of reactor can be improved.

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