Dipeptide Enzymatic Synthesis in a Two-Phase Membrane Reactor*

A. TRUSEK-HOLOWNIA and A. NOWORYTA

Institute of Chemical Engineering, Wroclaw University of Technology,
PL-50-373 Wroclaw, Poland
e-mail: trusek@iic.pwr.wroc.pl, noworyta@iic.pwr.wroc.pl

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The application of a two-phase system in enzymatic synthesis of bitter dipeptide ZAlaPheOMe was studied. Enzymatic conversion was accompanied by the reactant extraction in membrane contactor. Under experimental conditions, reaction kinetics and reactant mass transfer rates were evaluated and the main resistance of process was estimated. The Hatta number was used to the determination of the ratio of diffusion time to the reaction time. It was shown that the process ran in the kinetic region. Owing to a high enzyme activity and stability and the product extraction to organic phase, the substrate conversion degree 0.8 was achieved after 20 h of reaction time.

The enzymatic synthesis carried out in the presence of proteases, enzymes belonging to the hydrolase class, is one of the ways of peptide preparation. Due to the use of higher concentrations of slightly water-soluble substrates and the equilibrium shift towards products, it is advantageous to carry out this reaction in two-phase organic solvent—water system [1, 2]. In these two-phase systems the aqueous medium contains the biocatalyst and polar substrates while the hydrophobic reagents are dissolved in the organic phase (Fig. 1). Increase of substrate conversion is achieved by the hydrophobic substrate extraction from organic phase and the product extraction to organic phase [3—5]. The application of two-phase systems causes additional profits such as easiness of product separation and possibility to reuse the enzyme and nonconverted substrate.

Conventional two-phase equipment such as mixer-settler units or extraction column has got some disadvantages. The main drawbacks of column application are the limitations in interfacial surface area and the limitations in flow-rates of phases. With mixer-settler units there are problems due to emulsion breaking that in turn is stabilized by the protein used as catalyst [6]. Compared to these designs, a membrane phase contactor looks to be very promising [7, 8]. The porous membrane allows the two phases to be in contact without dispersion and subsequent emulsification and offers an extension of the unit surface area. The phases can be contacted at high flow-rates for both phases and there is no problem with flooding or loading [9, 10].

The membrane-based solvent extraction has got one disadvantage of an additional resistance in mass transfer. Its magnitude depends on membrane properties, extracted reactant features, and process conditions [11, 12]. When a hydrophobic membrane is used and the partition coefficient of extracted reactant (P_i) reaches a high value ($P_\mathrm{i} \gg 1$), it is possible that the membrane resistance becomes less important. The same is valid, when a hydrophilic membrane would be used and the reactants would have low values of the partition coefficients ($P_\mathrm{i} \ll 1$). In this case, the mass-transfer coefficient (K_org), given by eqn (*1*), reaches similar value as in extraction column under similar conditions [13].

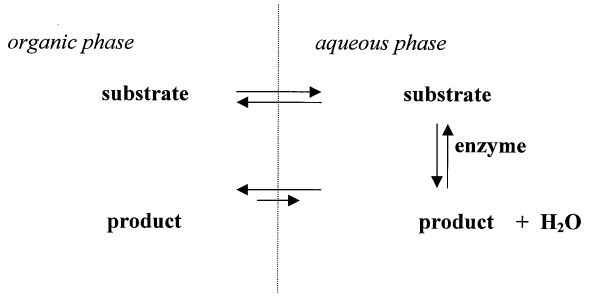

Fig. 1. Scheme of enzymatic catalysis in two-phase organic solvent—water system.

$$\frac{1}{K_{\mathrm{org,i}}} = \frac{P_\mathrm{i}}{k_{\mathrm{aq,i}}} + \frac{1}{k_{\mathrm{m,i}}} + \frac{1}{k_{\mathrm{org,i}}} \qquad (1)$$

where $K_{\mathrm{org,i}}$ is the overall mass-transfer coefficient of reactant "i" based on organic phase, $k_{\mathrm{org,i}}$ individual mass-transfer coefficient of reactant "i" based on organic phase, $k_{\mathrm{aq,i}}$ individual mass-transfer coefficient

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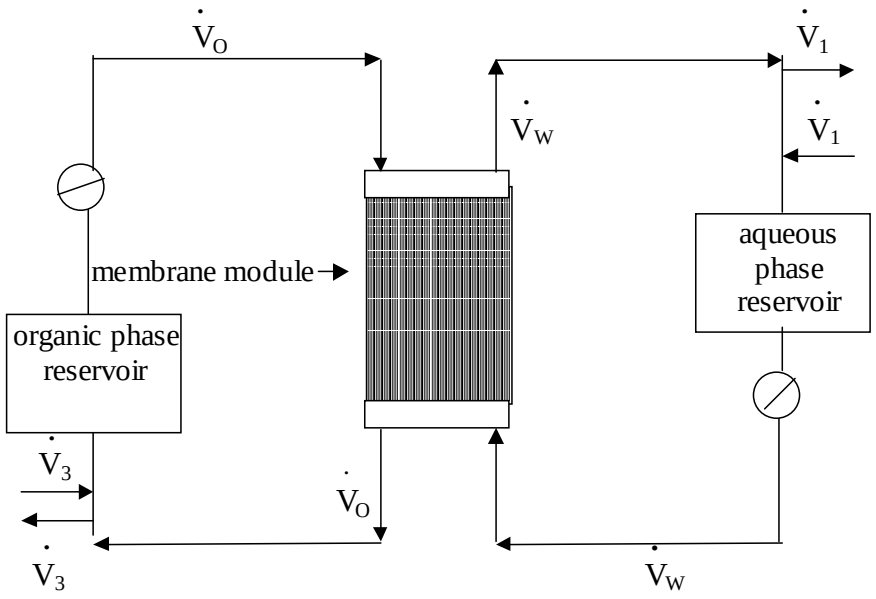

Fig. 2. Scheme of two-phase membrane reactor.

of reactant "i" based on aqueous phase, $k_{\mathrm{m,i}}$ individual mass-transfer coefficient of reactant "i" in membrane pores, and P_{i} partition coefficient of reactant "i".

Depending on the relative rates of reactant mass transfer and enzyme conversion, the process can run in kinetic, diffusion or mixed region. The Hatta number (Ha) determined by the ratio of diffusion time to the reaction time can be helpful in evaluations of reaction region [14]. For the first-order reaction, the Hatta number (Ha) takes the form

$$\mathrm{Ha} = \delta_{\mathrm{aq}} \cdot \sqrt{\frac{k_1}{D_{\mathrm{i}}}} = \frac{\delta_{\mathrm{aq}}}{D_{\mathrm{i}}} \cdot \sqrt{k_1 \cdot D_{\mathrm{i}}} = \frac{\sqrt{k_1 \cdot D_{\mathrm{i}}}}{k_{\mathrm{aq,i}}} \qquad (2)$$

where δ_{aq} is the thickness of diffusion film on aqueous side, k_1 reaction rate constant of the first-order kinetics, and D_{i} diffusion coefficient of reactant "i" in aqueous phase.

In this paper, the application of a two-phase membrane reactor to enzymatic peptide synthesis was investigated. The process was carried out in a membrane contactor prepared by the use of polypropylene capillaries. Under experimental conditions, reaction kinetics and reactants mass transfer rates were evaluated and the main resistance of process was estimated.

EXPERIMENTAL

ZAlaOH, PheOMe were purchased from Bachem (Bubendorf, Switzerland), crystalline thermolysin (EC 3.4.24.4) was obtained from Sigma (Missouri, USA). All used chemicals were of reagent grade.

Membrane bioreactor system was a prototype built in our laboratory. Membrane capillaries were supplied by Euro-Sep Company (Warszawa, Poland). Polypropylene capillaries (internal diameter equals 1.8 mm, pores length equals 0.4 mm, nominal pores size of 0.2 μm) were used. Membrane porosity was 0.7. Water contact angle of the capillaries equalled 94.3°.

The membrane module of a total volume of 47 cm^3 included 14 membrane capillaries of the effective length of 0.13 m. The external membrane area equalled 72 cm^2.

The reaction set up (Fig. 2) consists of an aqueous phase loop (100 cm^3 of tris-HCl buffer saturated with ethyl acetate, pH 7.0) and an organic phase loop (100 cm^3 of ethyl acetate saturated with tris-HCl buffer). Each phase was circulated with gear pump (ColeParmer Inc., Illinois, USA).

The measurements of the kinetic of ZAlaPheOMe synthesis according to the reaction

$$\mathrm{ZAlaOH} + \mathrm{PheOMe} \xrightleftharpoons{\text{thermolysin}} \mathrm{ZAlaPheOMe} + \mathrm{H_2O} \qquad (A)$$

were performed at 60 ℃ using 7.0 pH buffer (c(tris-HCl) = 0.05 mmol dm^{-3}), saturated with ethyl acetate. Initial concentrations of reactants in solutions varied between 2—60 mmol dm^{-3} for ZAlaOH, 25—1500 mmol dm^{-3} for PheOMe, and 5—20 μmol dm^{-3} for the enzyme. The reaction mixture was stirred in a batch reactor and samples were taken at appropriate intervals.

The reactant concentrations were measured with HPLC (LC Module, Waters Inc., Milford, USA) using a reverse phase RP 18 column (Nova-Pak Cartridge, Waters Inc., Milford, USA). A solution methanol—water—perchloric acid with the volume ratio 65:35:0.05 was used as a mobile phase and pumped with a flow-rate of 1 cm^3 min^{-1}. The absorbance was measured at λ = 254 nm.

Mass-transfer coefficient was estimated for each re-

actant supplied in the organic phase at the initial concentration of 40 mmol dm^{-3}. The phases were circulated at a flow-rate of 2×10^{-7}—5×10^{-7} m^3 s^{-1} and 1×10^{-7}—4×10^{-7} m^3 s^{-1}, respectively for aqueous and organic phase. The water phase was kept at 10 kPa overpressure to prevent the organic phase from penetrating through the membrane. The temperatures of both phases were kept at 60 °C. The concentrations of reactants in both phases were measured with HPLC.

Reactor Operation Procedure for ZAlaPheOMe Synthesis

The synthesis of ZAlaPheOMe was carried out in a two-phase membrane reactor. The aqueous (tubes) and organic (shell) phases were circulated at a flow-rate of 3.7×10^{-7} m^3 s^{-1} and 1.7×10^{-7} m^3 s^{-1}, respectively. The initial reactant concentrations were: 60 mmol dm^{-3} (ZAlaOH; organic phase) and 210 mmol dm^{-3} (PheOMe; aqueous phase). The enzyme concentration in aqueous phase was fixed at 15 μmol dm^{-3}. Samples from aqueous and organic phase were taken from the module outlet. The concentration of each reactant was measured with HPLC.

RESULTS AND DISCUSSION

Reaction Equilibrium in a Two-Phase System

For a two-phase system, the substrate conversion degree depends not only on the value of equilibrium constant and initial reactant concentrations but also on the partition coefficients P_i and phases volumes ratio β. In this case, the equilibrium (K) is given by the equation

$$K = \frac{\alpha_A^* \cdot c_{H_2O}}{c_{A0} \cdot (1 - \alpha_A^*) \cdot (M - \alpha_A^*)} \cdot \frac{(1 + P_A \cdot \beta) \cdot (1 + P_B \cdot \beta)}{\beta \cdot (1 + P_P \cdot \beta)} \qquad (3)$$

where c_{A0} is the initial concentration of substrate A, c_{H_2O} concentration of water, M initial reactants ratio, P_A, P_B, P_P partition coefficients of substrates A, B, and of product P, α_A^* equilibrium conversion degree of substrate A, and β phases volume ratio.

The values of the partition coefficients of reactants were estimated for the experimental system ethyl acetate—water (pH 7.0) at 60 °C. An influence of reactant concentration on the values of partition coefficients was observed. The partition coefficients reached the values of 1.5—5.0 for ZAlaOH at the concentrations of 5—60 mmol dm^{-3}, 0.3—0.7 for the PheOMe at 25—300 mmol dm^{-3}, and 273.5 for ZAlaPheOMe at 5—70 mmol dm^{-3}. The equilibrium constant K was determined on the basis of 8 reaction experiments carried out in buffer saturated with ethyl acetate as it was presented before [2]. The equilibrium constant was calculated to be 468.1.

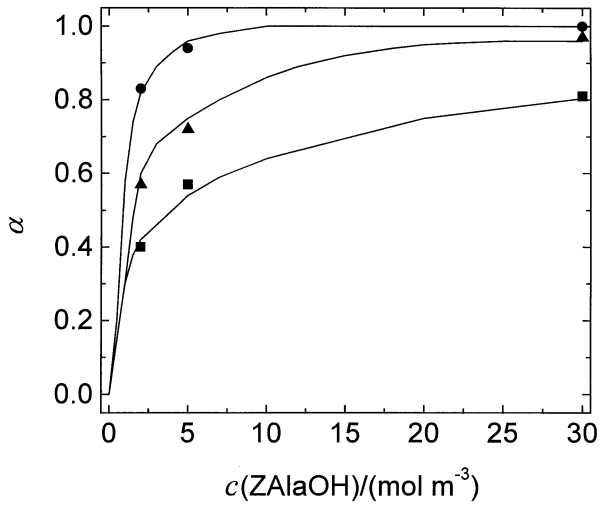

Fig. 3. Comparison of experimental (symbols) and calculated (lines) conversion degrees reached during ZAlaPheOMe synthesis reaction in two-phase ethyl acetate—water system ($\beta = 1$). ■ $M = 1$, ▲ $M = 2$, ● $M = 5$.

Eqn (*3*) was used to calculate the values of conversion degree for different reactant concentrations. These are shown in Fig. 3 together with the experimentally obtained values. It is worth noting that a high conversion degree was achieved even at low reactant concentrations. The conversion degree increased almost to unity for the concentration of ZAlaOH larger than 5 mmol dm^{-3} and of PheOMe larger than 10 mmol dm^{-3}. The high substrate conversions and the possibility to use higher reactant concentrations confirmed great efficiency of two-phase systems in peptide synthesis.

Reaction Kinetics

A ternary-complex mechanism with the random order of substrate binding was assumed for the description of kinetics of peptide synthesis by thermolysin. The reaction rate (v) was given by the following equation [15, 16]

$$v = \frac{k \cdot c_A \cdot c_B \cdot c_E}{K_{mA} \cdot K_{mB} + K_{mB} \cdot c_A + K_{mA} \cdot c_B + c_A \cdot c_B} \qquad (4)$$

where c_A, c_B is the concentration of substrate A, B, c_E concentration of enzyme, k reaction rate constant, and K_{mA}, K_{mB} equilibrium binding constant of substrate A and B.

The values of constants of eqn (*4*) were determined from the experiments carried out in ethyl acetate-saturated buffer in a wide range of reactant concentrations (2—60 mmol dm^{-3} (ZAlaOH), 25—1500 mmol dm^{-3} (PheOMe)). On the basis of 75 experimental points of the initial reaction rate using the computer program of nonlinear regression, the following values

were obtained: $k = 0.222$ s^{-1}, $K_{\mathrm{mA}} = 11.5$ mol m^{-3}, $K_{\mathrm{mB}} = 123.8$ mol m^{-3}. The reaction rates in the above-mentioned ranges of substrate concentrations and by the catalyst concentration range of 5—25 μmol dm^{-3} were approximately in the range of 10^{-5}—10^{-3} mol m^{-3} s^{-1}.

Interphase Mass Transfer

It has been shown by the additional, nonpublished investigations that a change of the concentrations of the reactants in inlet and outlet of the membrane module is very small. Hence, the constant value of the reactant concentrations along the membrane module could be assumed (by an insignificant mistake in further calculations).

The overall mass-transfer coefficient K_{org} under unsteady-state conditions could be evaluated from the following equation

$$-V_{\mathrm{org}} \cdot \frac{\mathrm{d}c_{\mathrm{org.i},t}}{\mathrm{d}t} = K_{\mathrm{org.i}} \cdot A \cdot (c_{\mathrm{org.i},t} - P_{\mathrm{i}} \cdot c_{\mathrm{aq.i},t}) \qquad (5)$$

that after integration by the use of the mass balance equation took a form

$$K_{\mathrm{org.i}} = \frac{1}{1 + \dfrac{P_{\mathrm{i}} \cdot V_{\mathrm{org}}}{V_{\mathrm{aq}}}} \cdot \frac{V_{\mathrm{org}}}{A \cdot t} \cdot \cdot \ln \frac{c_{\mathrm{org.i},0}}{c_{\mathrm{org.i},t} \cdot \left(1 + \dfrac{P_{\mathrm{i}} \cdot V_{\mathrm{org}}}{V_{\mathrm{aq}}}\right) - \left(c_{\mathrm{org.i},0} \cdot \dfrac{P_{\mathrm{i}} \cdot V_{\mathrm{org}}}{V_{\mathrm{aq}}}\right)} \qquad (6)$$

where V_{aq}, V_{org} is the volume of aqueous and organic phase, $c_{\mathrm{org.i},0}$, $c_{\mathrm{org.i},t}$ concentration in organic phase of reactant "i", initial and in time "t", and A membrane surface area.

The relation between the mass-transfer coefficients K_{org} and K_{aq} is given by the following equation

$$K_{\mathrm{aq,i}} = K_{\mathrm{org,i}} \cdot P_{\mathrm{i}} \qquad (7)$$

Fig. 4 presents an example of the concentration course observed for ZAlaOH. On the basis of these data, the value of overall mass-transfer coefficient K_{aq} was calculated using eqns (*6*) and (*7*) on the value of 4.6×10^{-6} m s^{-1}, with average relative error of 39.3 %. It was remarkable that the magnitude of mass-transfer coefficients of ZAlaOH and ZAlaPheOMe was mainly dependent on the aqueous phase flow-rate. It means that the organic phase resistance and membrane resistance had not significant influence on the mass transport rate in comparison with the aqueous phase resistance. The magnitude of mass-transfer coefficient of the second substrate PheOMe exhibited a stronger dependence on the organic phase flow-rate.

The rate of the mass transfer in membrane reactor can be intensified by the increase of interfacial surface area. The unit membrane surface area could be up to

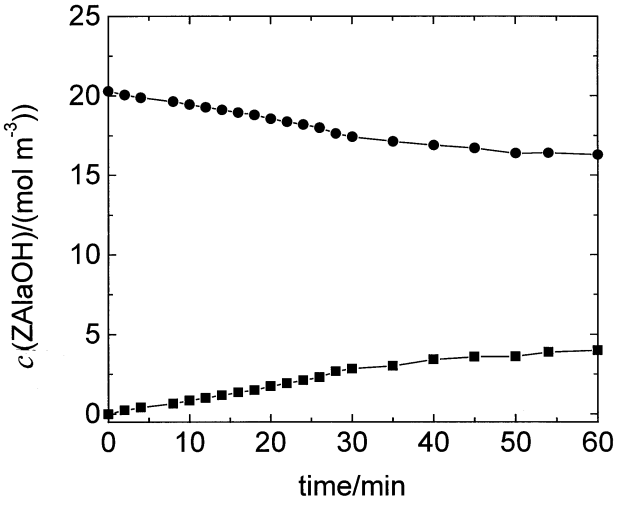

Fig. 4. Extraction process carried out at the flow-rate of 3.7×10^{-7} m^3 s^{-1} and 1.7×10^{-7} m^3 s^{-1} for aqueous (■) and organic (●) phase, respectively. Figure presents the concentration of ZAlaOH in module outlet.

10000 m^2 m^{-3} in commercial membrane contactors, that is a value much higher than the one reached in column or stirrer tank reactors.

Hatta Number Calculation

An analysis showed that the rate of the process depended mainly on the ZAlaOH concentration and the Hatta number was calculated on the basis of this component.

The concentration of PheOMe in aqueous phase was almost constant during the whole process owing to its high excess and higher affinity to water than to organic solvent. It means that an eventual transport of this reactant from organic phase could not have affected the overall process rate. The highly hydrophobic character of product resulted in a high value of its mass transport coefficient that was almost one order of magnitude higher than that of ZAlaOH. Hence, the product extraction rate could have a minimal effect on the process course.

In the experimental conditions used when the concentration of PheOMe in aqueous phase was almost stable and the constants presented the values of 0.222 s^{-1}, 11.5 mol m^{-3}, 123.8 mol m^{-3}, respectively for k, K_{mA}, and K_{mB}, eqn (*4*) could be simplified into the form of the pseudo-first order kinetics

$$v = k_1 \cdot c_{\mathrm{aq,A}} \qquad (8)$$

where $k_1 = 7.7 \times 10^{-5}$ s^{-1} for 15 mol m^{-3} > $c_{\mathrm{aq,A}}$ > 10 mol m^{-3} and 1.2×10^{-4} s^{-1} for 10 mol m^{-3} > $c_{\mathrm{aq,A}}$ > 1 mol m^{-3}.

The diffusion coefficient (D_{i}) of ZAlaOH in water

was calculated from the equation

$$D_{\mathrm{i}} = \frac{R \cdot T}{6 \cdot \pi \cdot \eta \cdot N \cdot r_{\mathrm{i}}} \qquad (9)$$

where R is the gas law constant, T temperature, η viscosity of water at 60 °C = 4.688×10^{-4} kg m^{-1} s^{-1}, N Avogadro's constant, and r_{i} radius of molecule, $r_{\mathrm{A}} = 0.192 \times 10^{-9}$ m.

The value of the diffusion coefficient of ZAlaOH was equal to 2.6×10^{-9} m^2 s^{-1}.

By the assumption that the value of $k_{\mathrm{aq,A}}$ is much smaller than the values of $(k_{\mathrm{org,A}} \cdot P_{\mathrm{A}})$ and $(k_{\mathrm{m,A}} \cdot P_{\mathrm{A}})$, eqn (*10*) could be simplified to the form (*11*).

$$\frac{1}{K_{\mathrm{aq,i}}} = \frac{1}{k_{\mathrm{aq,i}}} + \frac{1}{k_{\mathrm{m,i}} \cdot P_{\mathrm{i}}} + \frac{1}{k_{\mathrm{org,i}} \cdot P_{\mathrm{i}}} \qquad (10)$$

$$\frac{1}{K_{\mathrm{aq,A}}} \cong \frac{1}{k_{\mathrm{aq,A}}} \qquad (11)$$

In this case, at the flow-rate 3.7×10^{-7} m^3 s^{-1} for aqueous phase and 1.7×10^{-7} m^3 s^{-1} for organic phase, $k_{\mathrm{aq,A}}$ was equal to 4.6×10^{-6} m s^{-1}. The Hatta number calculated from eqn (*2*) reached the values of 0.1 for $k_1 = 7.7 \times 10^{-5}$ s^{-1} and 0.12 for $k_1 = 1.2 \times 10^{-4}$ s^{-1}.

Process of Enzymatic Synthesis of ZAlaPheOMe in Two-Phase Membrane Reactor

In the chosen solvent system of ethyl acetate—water, the organic phase performs the function of hydrophobic substrate (ZAlaOH) reservoir while the aqueous phase is the hydrophilic substrate (PheOMe) and catalyst reservoir. The reactant extraction from/to organic phase accompanies the reaction running in aqueous phase. Taking into consideration the properties of extracted reactant (especially of ZAlaOH), a hydrophobic membrane with pores filled by organic phase was chosen.

The process ran periodically under unsteady conditions. The samples were taken from the module outlets for both phases. Fig. 5 presents the course of reactant concentrations in aqueous and organic phases. The experimental data show that:

1. Eqn (*4*) described the process rate in this two-phase system with a good accuracy (the average error of 18.7 %). This confirmed that the process was conducted in the kinetic region. The enzyme stability was similar to the one reached in saturated buffer since the interfacial inactivation of enzyme was not observed.

2. It was found that during the process, interfacial equilibrium was reached. It means that the structure and properties of membrane contactor ensured a very fast mass transfer. This would allow to increase the volume of reaction phase that would lead to higher process efficiency.

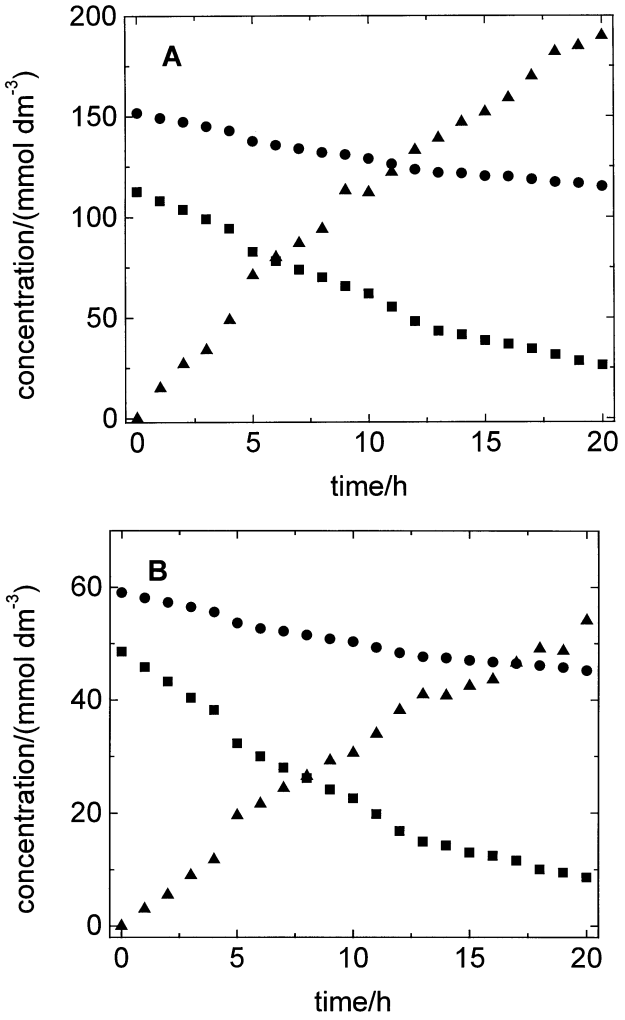

Fig. 5. Concentrations of ZAlaOH (■), PheOMe (●), and ZAlaPheOMe (▲) in aqueous (A) and organic (B) phases during ZAlaPheOMe synthesis in membrane contactor at $\beta = 1$.

3. The linearity of the changes of the product concentration in organic phase confirmed the high value of mass-transfer coefficient.

4. A product concentration of 55 mmol dm^{-3} was obtained after 20 h of the synthetic reaction that corresponded to the conversion degree of 0.8.

SYMBOLS

A	membrane surface area	m^2
c	concentration	mol m^{-3}
D	diffusion coefficient	m^2 s^{-1}
K	equilibrium constant	
K_{aq}, K_{org}	overall mass-transfer coefficient based on aqueous and organic phase	m s^{-1}
K_{mA}, K_{mB}	equilibrium binding constant	mol m^{-3}
k, k_1	reaction rate constant	s^{-1}

k_{aq}, k_m, k_{org}	individual mass-transfer coefficient in aqueous phase, in membrane and in organic phase, respectively	m s^{-1}
M	initial reactants ratio ($M = n_{B0}/n_{A0}$)	
N	Avogadro's constant	mol^{-1}
P	partition coefficient ($P_i = c_{org,i}/c_{aq,i}$)	
R	gas law constant	J mol^{-1} K^{-1}
r	radius of molecule	m
T	temperature	K
V	volume	m^3
α^*	equilibrium conversion degree	
β	phases volume ratio ($\beta = V_{org}/V_{aq}$)	
δ	thickness of diffusion film	m
η	dynamic viscosity	kg m^{-1} s^{-1}

Subscripts

A	substrate ZAlaOH
B	substrate PheOMe
P	product ZAlaPheOMe
aq	aqueous phase
i	reactant
m	membrane
org	organic phase
t	time
0	initial

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