

Mathematical modelling of ester synthesis by lipase in biphasic system

Tadeusz Antczak^{*}, Dariusz Hiler, Alina Krystynowicz, Stanislaw Bielecki,
Edward Galas

Institute of Technical Biochemistry, Technical University of Lodz, 90-924 Lodz, Stefanowskiego 4 / 10, Poland

Abstract

The invented mathematical model of ester synthesis in a biphasic system using the immobilised *Mucor circinelloides* lipase, situated in organic phase, was experimentally verified by butyl oleate, oleyl oleate, sucrose oleate and sucrose caprylate synthesis. The model took into consideration the fact revealed in experiments that water being a by-product of a reaction, created a separate phase thus influencing a value of equilibrium constant in the organic phase (K_O). The applied mathematical model makes it possible to determine with high accuracy the yield of ester synthesis that can be obtained in the reaction poststational state. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Ester synthesis; Lipase; Biphasic system; Mathematical modelling

1. Introduction

In ester enzymatic synthesis water is one of the reaction mixture constituents and products of enzymatic ester synthesis in organic solvents milieu [1,2]. If esters are synthesised in apolar organic solvents, saturated with water, its portion emerging from the

reaction creates another phase whose volume may markedly influence the final reaction yield.

2. Theory

The esterification reaction can be presented in the form of a scheme:

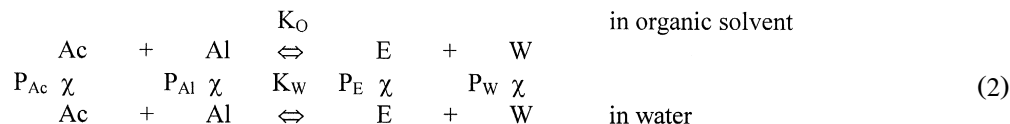


According to the mathematical model presented in previous works [3–6] in enzymatic reaction milieu,

^{*} Corresponding author.

E-mail address: tad45an@snack.p.lodz.pl (T. Antczak).

in a biphasic system, reagents are separated between water and organic phases, which is illustrated by the



where Al is alcohol, Ac acid, E ester, W water; K_O , and K_W are reaction equilibrium constants in organic and water phases, respectively; P_{Ac} , P_{Al} , P_E , P_W are partition coefficients between organic and water phases for acid, alcohol, ester and water, respectively.

Partition coefficients (P) of substrates and products (reagents, R) between immiscible phases are expressed by the following relationship:

$$P_{(Ac,Al,E,W)} = \frac{[R]_O^S}{[R]_W^S} \quad (3)$$

where $[R]_O^S$ and $[R]_W^S$ are the concentration of each reagent in organic and water phase respectively, in saturation state, $[\text{mol dm}^{-3}]$.

Equilibrium constant of esterification reaction in a biphasic system, diphasic constant (K_{BI}), has the following form [3]:

$$K_{BI} = \frac{[E]_T [W]_T}{[Ac]_T [Al]_T} \quad (4)$$

where $[E]_T$, $[W]_T$, $[Ac]_T$, $[Al]_T$ are total reagent concentration (of ester, water, acid and alcohol, respectively) in biphasic system $[\text{mol dm}^{-3}]$.

A biphasic reaction system is also characterised by the coefficient of phase volume ratio (A):

$$A = \frac{V_O}{V_W} \quad (5)$$

where V_O , V_W is the volume of organic and water phase, $[\text{dm}^3]$.

following scheme:

The mass balancing equation of acid, alcohol, ester and water had the following form [5].

$$[R]_T V_T = [R]_O V_O + [R]_W V_W \quad (6)$$

where $[R]_T$ is the total concentration of a reagent in the system, V_T the total system volume; $[R]_O$, $[R]_W$ are concentrations of a reagent in organic and water phase, respectively. Concentration is expressed in $[\text{mol dm}^{-3}]$.

The model given by Martinek [3] referred to a system in which the enzyme was dissolved in water phase and esterification reaction took place at phase boundary. This model did not take into account water increase in the system during the esterification. It assumed that the value of equilibrium constant in organic phase (K_O) is a parameter that is not affected by the water phase volume (V_W). A biphasic constant according to Martinek had the following form:

$$K_{BI} = K_O \frac{P_{Ac} P_{Al} (1 + P_E A) (1 + P_W A)}{P_E P_W (1 + P_{Ac} A) (1 + P_{Al} A)} \quad (7)$$

It was stated that this model does not represent the ester synthesis, catalysed by lipase *Mucor* in biphasic system, well enough. The reason is that K_{BI} values, calculated according to formula formula (7), differ significantly from the values empirically determined for different systems such as: in oleyl oleate (in naphthyl ether–water milieu) and sucrose caprylate (in a di-*n*-pentyl ether–water) synthesis. In our investigations a lipase preparation in immobilised form was situated in organic phase and water secreted during the esterification reaction diffused to a

model water phase increasing its volume. That is why the model was modified as follows.

It was assumed that the total volume of biphasic system (V_T) is a sum of volume fractions of individual phases and amounts to 1 dm^3 , which can be expressed:

$$V_T = V_O + V_W = 1 \text{ dm}^3 \quad (8)$$

Substituting the phase volume ratio (A) it can be calculated:

$$V_O = \frac{A}{1+A} \quad (9)$$

and

$$V_W = \frac{1}{1+A} \quad (10)$$

The assumptions were as follows:

1. The total system volume (V_T) does not change during the reaction.
2. Because the organic phase is saturated with water, water secreted during the reaction diffuses into water phase, thus increasing its volume, which results in a decrease of organic phase volume.

The volumes of the organic phase (V_O) and water phase (V_W) in an equilibrium state of reaction are as follows:

$$V_O = V_O^0 - V_S \quad (11)$$

$$V_W = V_W^0 + V_S \quad (12)$$

$$K_{BI} = f(A) \frac{P_{Ac} P_{Al}}{P_E P_W [W]_O} \times \frac{[1 + P_E A + V_S(1 - P_E)(1 + A)][(1 + P_W A)[W]_O + P_W(1 + A)(V_S + V_N[W]_N)]}{[1 + P_{Ac} A + V_S(1 - P_{Ac})(1 + A)][1 + P_{Al} A + V_S(1 - P_{Al})(1 + A)]} \quad (14)$$

On the other hand taking into consideration the overreacting of substrates (acid and alcohol), Eq. (13) presenting water content in the system as well

where V_O^0 , V_W^0 are volumes of organic and water phases respectively, at the beginning of reaction, V_S the volume of water secreted in ester synthesis reaction.

Total water content in biphasic system (W_T), expressed in moles is presented in a form of material balance equation:

$$W_T = V_O[W]_O + V_W[W]_W + V_S[W]_W + V_N[W]_N = V_T[W]_T \quad (13)$$

where the individual sum components designate: $V_O[W]_O$, water in organic phase, [mol]; $V_W[W]_W$, water in water layer, [mol]; $V_S[W]_W$, water secreted during the reaction, [mol]; V_N , volume of essential enzyme bound water layer, [dm^3]; $[W]_N$, water concentration in essential enzyme bound water layer [mol dm^{-3}].

For the model ester synthesis catalysed by *M. circinelloides* in biphasic systems of different phase volume coefficients (A), it was empirically revealed that the value of equilibrium constant in organic phase (K_O) depends on the phase volume ratio (A), applied in the reaction. Then, the relationships $K_O = f(A)$ for the investigated different ester synthesis systems were presented in form of mathematical equations. Some of them are presented in Fig. 1. It is supposed that experimentally determined coefficients of these equations describe the influence of the reaction milieu on a lipase molecule [7]. Taking into consideration that K_O is a function of A and substituting the relationships (3), (5), (6), (8), (9) and (13), to Eq. (4) we get the following equation:

as assuming that the number of water moles resulting from the reaction ($V_S[W]_W$) is equal to the number of ester moles (E_T), the equation of esterification

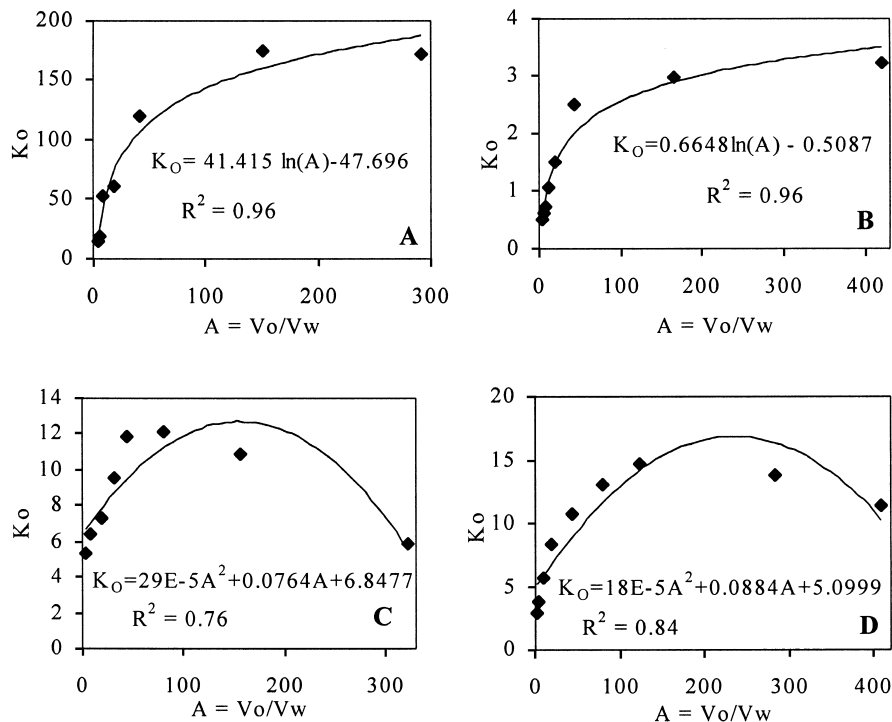


Fig. 1. Effect of phase volume ratio (A) on the equilibrium constant in organic phase (K_O), for synthesis of: (A) butyl oleate, (B) oleyl oleate, (C) sucrose caprylate, (D) sucrose oleate (Section 3.2). Experimental values are presented as points, and the continuous line corresponds to values calculated according to the determined function $K_O = f(A)$.

equilibrium constant K_{BI} in biphasic system (4) can be expressed by the equation form (15). It is assumed in the formula that the total volume of the system is not changed and amounts to 1 dm^3 .

$$K_{BI} = \frac{E_T(V_O[W]_O + V_W[W]_W + E_T + V_N[W]_N)}{(Ac_T^0 - E_T)(Al_T^0 - E_T)} \quad (15)$$

where: E_T is the number of ester moles in the system, Ac_T^0 , Al_T^0 , total amount of acid and alcohol moles respectively, used in the reaction.

Taking into consideration that the same numbers of substrate moles were used in the reaction and designating them with symbol S (i.e. $[Ac]_T^0 = [Al]_T^0 = [S]$) and applying relationships (9) and (10), Eq.

(15) was transformed to a quadratic equation (16). One of the solutions of this equation enables forecasting of ester synthesis yield in poststational state. For this equation value K_{BI} is calculated from the relationship (14).

$$([E]_T)^2(K_{BI} - 1) - [E]_T \left\{ \frac{[W]_W(P_W A + 1)}{A + 1} + [W]_N V_N + 2[S]K_{BI} \right\} + K_{BI}[S]^2 = 0 \quad (16)$$

The introduced model was verified in investigations on the synthesis of different esters in biphasic system. Some of them are presented below.

3. Materials and methods

3.1. Biological material

The *M. circinelloides* lipase in a form of dehydrated mycelium was prepared as described in works [8,9].

3.2. Determination of relationship K_O in function of A

Ester synthesis was executed in minireactors placed on a shaker. Each minireactor contained 1 mmol of acid, 1 mmol of alcohol and solvent (previously saturated with water) and water in proportions according to the determined phase volume ratio (A). Then, 50 mg of lipase preparation in polyethylene basket, which ensured good mass transfer and prevented lipase penetration into water phase, was placed in each minireactor. Total volume of the reaction mixture was equal to 5 cm³. Aliphatic alcohols ester synthesis was carried out in naphthyl ether milieu. Esters of sucrose — in di-*n*-pentyl ether, in the milieu where acid and sucrose react at mole relation 1:1 (sucrose:esters), for 144 h at 50°C and 250 rpm.

The efficiency of ester synthesis was determined by titration [9] and the values of equilibrium constant in K_O solvent milieu were calculated.

3.3. Determination of partition coefficients, $P_{Ac, Al, E}$

Coefficients of reagent partition were determined by measurement of their concentration, in the saturated state, in organic solvents and water at 50°C. Concentration of dissolved acids was determined by titration [9]. The concentration of alcohol and oleic acid esters was determined by GC Carlo-Erba (column 30 m, stationary phase DB-17, film thickness 0.25 μm, temperature 60–300°C). The concentration of sucrose and sucrose esters was determined using HPLC Gold Beckman (column ODS: 250 × 5.6 mm, elution with mixture: acetonitrile–water (8:2), refractometric detector). Water concentration was determined according to Karl Fischer method (Mettler DL18).

Modelling was done using the Mathematica 3.0. program.

4. Results and discussion

Ester synthesis in a non-water system always runs at a water–solvent phase boundary. Water phase includes water introduced into organic solvent, water secreted during the reaction or water present in enzyme preparation as essential enzyme-bound water. The synthesis of higher fatty acid esters of alcohols at high initial and final reagent concentrations (from 2 M to 3 M) is a source of water secreted in an apolar phase, which creates the biphasic system. The phase volume ratio amounts to $A \approx 20$ –40.

It has been noticed that water phase volume in the system affects considerably the final efficiency of the reaction catalysed by immobilised in situ intracellular *M. circinelloides* lipase. In order to investigate this effect the models of selected ester synthesis were made in biphasic systems with different initial volume of water phase (Section 3.2).

The results in the form of relationship between the equilibrium constant K_O and the value of phase volume ratio (A) in the reaction poststational state are shown in Fig. 1 (A–D). It was stated that in the case of ester synthesis of fatty acids and aliphatic

Table 1
Values of the mathematical model factors, applied for calculations in Eq. (16)

Model factor	Butyl oleate	Oleyl oleate	Sucrose caprylate	Sucrose oleate
P_{Ac}^a	5800	5800	961	3000
P_{Al}^a	11.68	5000	0.00021	0.00021
P_{W}^a	0.0001	0.0001	0.00437	0.00437
P_E^a	7500	16000	0.0015	0.0023
A^a	28	29	40	40
V_S^a	0.0357	0.0346	0.0244	0.0242
$[W]_O^a$	0.0232	0.0232	0.24289	0.24289
$[W]_W^b$	55.55	55.55	55.55	55.55
$[W]_N^b$	55.55	55.55	55.55	55.55
V_N^b	1×10^{-8}	1×10^{-8}	1×10^{-8}	1×10^{-8}
$[S]^a$	2	2	2	2
$K_O = f(A)^a$	Fig. 1A	Fig. 1B	Fig. 1C	Fig. 1D

^aExperimental values.

^bArbitrary values.

Table 2
Yield of ester synthesis in biphasic system (substrate concentration $[S] = 2 \text{ mol} \times \text{dm}^{-3}$)

Ester	A	Ester synthesis yield, E (%)	
		Theoretical ^a	Experimental ^b
Buthyl oleate	28	99.3	96.2
Oleyl oleate	29	94.6	94.0
Sucrose caprylate	40	67.4	67.8
Sucrose oleate	40	61.4	67.4

^aCalculated according to model (16).

^bE% values determined experimentally were used for calculation of water volume secreted during the reaction, which was applied in Eq. (16), e.g. for $E\% = 96.2$ and $[S] = 2 \text{ M}$, value of $V_s = [S] E\% 0.018/100\% = 0.0357 \text{ dm}^3$.

alcohols, Fig. 1(A,B), function $K_O = f(A)$ is monotonously growing, which suggests a need of water removal during the reaction to maintain high degree of substrates conversion. In function $K_O(A)$ obtained for sucrose ester synthesis there is a maximum within the range of limit values of coefficient A, which suggests the need of controlling this parameter during the reaction.

Determination of a function $K_O = f(A)$ describing the influence of a biphasic system on synthetic activity of *Mucor* lipase enabled the application of a mathematical model (16) for theoretically forecasting the yield of esterification reaction. Coefficient values applied for model calculations are included in Table

1. The calculations of forecasted yields of ester synthesis confirmed the experimental results. Some examples are given in Table 2.

Figs. 2 and 3 show examples of computer simulation illustrating the simultaneous effect of two model components on the yield of sucrose caprylate synthesis reaction. The ranges of numbers of investigated model component variables are shown under the diagrams.

On the basis of the analysis of the effect of all model components it was stated that the yield of this ester synthesis depends on coefficients values: phase volume ratio (A), partition of products (P_E , P_W), alcohol partition (P_{Al}) and water concentration in water phase ($[W]_W$).

The partition coefficients of products P_E and P_W affect ester synthesis yield if their value is $\ll 1$. If their value is decreased ester synthesis yield is higher (Fig. 2).

The decrease of water concentration in water phase ($[W]_W$), which can be achieved by, e.g. making a mixture of water and well soluble substances, slightly increases the forecasted yield of sucrose caprylate synthesis (Fig. 3a). The decrease of water concentration by 10 times, which is difficult to get, results in 10% rise in ester synthesis yield.

According to model calculations (Fig. 3b), a considerable rise in ester synthesis yield can be achieved by increasing sucrose solubility in organic milieu (increased P_{Al}). In the work of Klibanov [1] it was

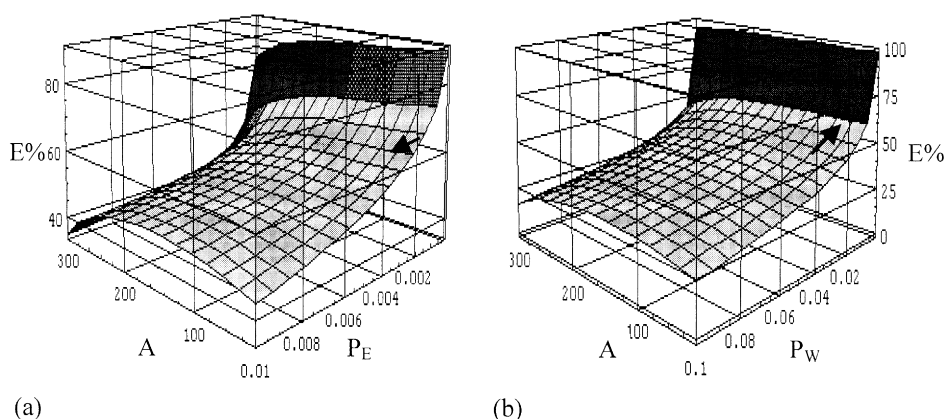


Fig. 2. Modelling of the effect of coefficients A and P_E (a) as well as A and P_W (b) on sucrose caprylate synthesis yield (initial concentration of substrates $[S] = 2 \text{ mol dm}^{-3}$). Ranges of coefficient values in model investigations: A (25–300), P_E (0.0001–0.01), P_W (0.00001–0.1). Arrows indicate points confirmed experimentally.

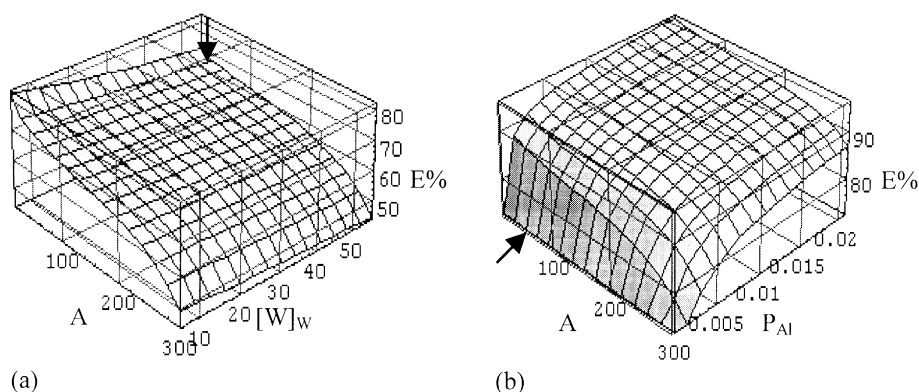


Fig. 3. Modelling of the effect of coefficients A and $[W]_w$ (a) as well as A and P_{Al} (b) on sucrose caprylate synthesis yield (initial concentration of substrates $[S] = 2 \text{ mol dm}^{-3}$). Ranges of coefficient values in model investigations: A (10–300), $[W]_w$ (5.55–55.55), P_{Al} (0.00021–0.021). Arrows indicate points confirmed experimentally (Table 2).

stated that the energy of binding between an enzyme and a substrate is the main source of energy for enzymatic catalysis as well as a source of huge rate enhancement afforded by enzyme. The substrate must first undergo desolvation to enable its binding in an enzyme active centre. The more energetically preferred is such dissolving the more net binding energy appears in the systems a result of catalysis. In other words during the sucrose ester synthesis low solubility of sucrose substrates in an apolar milieu limits the progress of lipase-catalysed synthesis, which is confirmed by forecasted synthesis yield calculated by means of the mathematical model suggested in this work. This problem is solved in present investigations by the application of sucrose derivatives as substrates for more water-soluble lipases in apolar milieu [10].

5. Summary

The applied mathematical model was verified experimentally for the synthesis of many esters catalysed by *M. circinelloides* lipase. Apart from the esters mentioned in the work, they were stearates: of propyl, butyl, and oleyl as well as sucrose myristate and glucose caprylate, myristate and oleate. In all cases the applied model enabled theoretical calculation of ester synthesis yield with high accuracy. It can be used for forecasting of synthesis yield in

biphasic systems. All model components have real physical dimensions and only the coefficients of the equation $K_O = f(A)$ should be defined experimentally for a given reaction system. The presented mathematical model makes it possible to identify the reaction parameters that limit the yield of ester synthesis in a biphasic system, thus indicating the directions to increase its yield.

List of symbols

E_T	amount of generated ester [mol]
Ac_T^0	number of acid moles used in reaction [mol]
Al_T^0	number of alcohol moles used in reaction [mol]
W_T	total amount of water in a biphasic system [mol]
K_{BI}	apparent equilibrium constant (in biphasic system) [–]
K_O	equilibrium constant in organic phase [–]
K_W	equilibrium constant in water phase [–]
P_{Ac}	partition coefficients between organic and water phases for acid [–]
P_{Al}	partition coefficients between organic and water phases for alcohol [–]
P_E	partition coefficients between organic and water phases for ester [–]
P_W	partition coefficients between organic and water phases for water [–]
A	phase volume ratio [–]

V_O^0	organic phase volume at the beginning of reaction [dm ³]
V_W^0	water phase volume in biphasic system in the beginning of reaction [dm ³]
V_O	organic phase volume during the reaction [dm ³]
V_W	water phase volume at the beginning of reaction [dm ³]
V_N	an essential lipase-bound water layer volume [dm ³]
V_T	total system volume [dm ³]
V_S	water volume secreted during the reaction [dm ³]
$[W]_O$	concentration of water in organic phase [mol dm ⁻³]
$[W]_W$	concentration of water in water phase [mol dm ⁻³]
$[W]_N$	concentration of water in an essential lipase-bound water layer [mol dm ⁻³]
$[W]_T$	total water concentration in the system [mol dm ⁻³]
$[S]$	concentration of substrates (acid concentration equal to alcohol conc.) [mol dm ⁻³]
$[E]_T$	total ester concentration in the system [mol dm ⁻³]
$[Ac]_T$	total acid concentration in the system [mol dm ⁻³]
$[Al]_T$	total alcohol concentration in the system [mol dm ⁻³]

$[Al]_T^0$	total, initial concentration of alcohol in the system [mol dm ⁻³]
$[Ac]_T^0$	total, initial concentration of acid in the system [mol dm ⁻³]

Acknowledgements

The authors gratefully acknowledge the financial support of the State Committee for Scientific Research (KBN) for this work under Grant 6PO4B00311.

References

- [1] A.M. Klibanov, Trends Biotechnol. 15 (1997) 97.
- [2] P.J. Halling, Enzyme Microb. Technol. 16 (1994) 178.
- [3] K. Martinek, A.N. Semenov, I.V. Berezin, Biochim. Biophys. Acta 658 (1981) 76.
- [4] K. Martinek, A.N. Semenov, J. Appl. Biochem. 3 (1981) 93.
- [5] A.N. Semenov, Yu.L. Khamelnitski, I.V. Berezin, K. Martinek, Biocatalysis 1 (1987) 4.
- [6] A.N. Semenov, Prikl. Biochim. Mikrobiol. 30 (1994) 302.
- [7] T. Antczak, A. Krystynowicz, D. Hiler, S. Bielecki, E. Galas, Biotechnologia 44 (1999) 153.
- [8] T. Antczak, A. Krystynowicz, E. Galas, Biotechnologia 29 (1995) 82.
- [9] U. Antczak, J. Góra, T. Antczak, E. Galas, Enzyme Microb. Technol. 13 (1991) 589.
- [10] I. Ikeda, A.M. Klibanov, Biotechnol. Bioeng. 42 (1993) 788.