KINETICS OF GLYCEROL BIOTRANSFORMATION TO DIHYDROXYACETONE BY IMMOBILIZED *Gluconobacter oxydans* AND EFFECT OF REACTION CONDITIONS

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Biotransformation of glycerol to 1,3-dihydroxyacetone was carried out in an isothermal isochoric batch reactor with *Gluconobacter oxydans* immobilized in poly(vinyl alcohol) gel capsules. The reaction course was described with a three-step kinetic model. Two reaction schemes were proposed and compared with 8 kinetic experiments at 25 °C. The experimental dependences of glycerol and dihydroxyacetone concentrations on reaction time were simulated very well by the autocatalytic model. The effects of reaction temperature and initial concentrations of yeast extract and glycerol were studied. Temperature 25–30 °C, initial yeast extract concentration 2–4 g l^{-1} and initial glycerol concentration 20–50 g l^{-1} were found as optimal. The determined rate constants can be used to advantage for industrial production of dihydroxyacetone from glycerol.

Keywords: Dihydroxyacetone; Glycerol; Biotransformations; Kinetics; Immobilized microorganisms; Biotechnology.

Today microbial and chemical technologies compete. Some products cannot be synthetically produced economically enough and some chemical procedures require many security precautions. In these cases the microbial methods are preferred due to their highly specific course and slight influence on environment. This holds also for the sythesis of 1,3-dihydroxyacetone (D) from glycerol (G)[1].

The strictly aerobic microorganism *Gluconobacter oxydans* (*G. oxydans*), which is able to dehydrogenate various types of monosaccharides, alcoholic sugars, aliphatic and cyclic alcohols, is used for industrial production of D from G[1–12]. D is presently produced mostly by biotransformation (biological oxidation) of G with free cells of *G. oxydans*. D is used as a component of bronzing lotions and as a raw material for the production of pharmaceuticals. In the industrial fermentation the fed-batch reactors are often used, which can reduce the effect of the substrate inhibition on the process pro-

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ductivity[1]. The most modern biotechnologies utilize the immobilisation of microbial cultures. Its advantages are an increase in biocatalyst concentration and improvement of technological properties. There are many immobilisation techniques. The most used ones are immobilisation in a biofilm on the surface of a suitable carrier and encasement into semipermeable capsules (encapsulation), e.g. by the LentiCats® technology[13]. The last technique, using the poly(vinyl alcohol) capsules, was used in this work, which deals with

– the formal kinetics of the biotransformation of G to D by encapsulated *G. oxydans* in the isothermal isochoric batch reactor. This description is based on experimental dependences of G and D mass concentrations (*G* and *D*, both in g l^{-1}) on reaction time *t*, and

– the influence of initial concentrations of substrate and yeast extract and temperature of the reaction mixture.

THEORY

Because the reaction mixture during the G biotransformation to D is heterogeneous, the total time course must be a superposition of consecutive steps, such as diffusion and adsorption of G and D on the LentiCats® capsules (L), and biochemical transformation of G to D. It is necessary to find the rate-determining (slowest) step. Therefore, the rate of G and D diffusion through the L poly(vinyl alcohol) membrane (PVA) into the capsules and back into the reactor volume was preliminary determined. It was found that both processes are much faster than the biochemical transformation proper. Therefore it can be assumed that the slowest step in the whole process is the chemical reaction[14]. If the biotransformation proceeds well, glyceric acid (A) is also formed[7] besides D. Therefore the reaction mixture must be maintained at constant pH value with an alkali. Glyceric acid is formed immediately from the reaction start, when no D is yet produced. It can be assumed that A is formed in a side (parallel) reaction besides the oxidation of G to D.

Another side step is the consumption of G by the internal metabolism of the microorganism in cytoplasm. G serves as the structural material and energy source for *G. oxydans*. In the following models, the product of this metabolism is marked as M. It was verified that for the simplest reaction model the processes producing A and M can be approximately described by the first-order reaction with respect to G [10,14].

All our experimental dependences *G* and *D* vs *t* obtained in the batch reactor are of sigmoidal form (Figs 2, 3, 5, 7). Therefore, both kinetic models

mentioned below were first tested for the existence of the inflexion point on the theoretical dependences *G* and *D* vs *t*.

As the first reaction scheme was proposed the simplest model optimally expressing the physical principle of the reaction.

Catalytic Model

This model presumes three side steps.

$$\mathrm{G} + \mathrm{E} \xrightarrow{\mathrm{O_2},\ k_1} \mathrm{D} + \mathrm{E} \quad (1) \qquad \mathrm{G} \xrightarrow{\mathrm{O_2},\ k_2} \mathrm{M} + \mathrm{E} \quad (2) \qquad \mathrm{G} \xrightarrow{\mathrm{O_2},\ k_3} \mathrm{A} + \mathrm{E} \quad (3)$$

In the first step (*1*) G reacts with the enzymes E included in encapsulated *G. oxydans* under D production. The second step (*2*) expresses formally the growth of *G. oxydans* and the gain of energy at the expense of G. In the third step (*3*), the experimentally detected glyceric acid A is generated. By rather complicated mathematical analysis of the differential kinetic equations describing this model,

$$-\mathrm{d}G/\mathrm{d}t = G(k_1E + k_2 + k_3)$$

$$\mathrm{d}E/\mathrm{d}t = (k_2 + k_3)G$$

$$\mathrm{d}A/\mathrm{d}t = k_3GE\ ,$$

it could be demonstrated, that the dependences of *G* and *D* vs *t* have no point of inflexion and thus cannot simulate the obtained experiments. Therefore, this model is unsuitable.

The second, formal, purely statistical reaction scheme is the autocatalytic model, treated in the next paragraph.

Autocatalytic Model

In this model G reacts formally with E under production of not only A, D and M but also regeneration of E.

$$\mathrm{G} + \mathrm{E} \xrightarrow{\mathrm{O_2},\ k_\mathrm{D}} 2\ \mathrm{E} + \mathrm{D} \quad (4) \qquad \mathrm{G} + \mathrm{E} \xrightarrow{\mathrm{O_2},\ k_\mathrm{M}} 2\ \mathrm{E} + \mathrm{M} \quad (5) \qquad \mathrm{G} + \mathrm{E} \xrightarrow{\mathrm{O_2},\ k_\mathrm{A}} 2\ \mathrm{E} + \mathrm{A} \quad (6)$$

Let *G*, *E*, *D*, *M* be the actual mass concentrations of G, E, D, M (in g l^{-1}), *g*, *e* initial mass concentrations of G, E, and *I* stands for actual concentra-

tion of any given reagent. Then for G and D following differential kinetic equations in the batch reactor can be written

$$-\mathrm{d}G/\mathrm{d}t = (k_\mathrm{D} + k_\mathrm{M} + k_\mathrm{A})GE = \mathrm{d}E/\mathrm{d}t \tag{7}$$

$$\mathrm{d}D/\mathrm{d}t = k_\mathrm{D}GE \tag{8}$$

where the rate constants k, k_D and k_M are in l g^{-1} s^{-1}. After introduction of relative concentrations $\varphi_\mathrm{i} = I/g$, we have the kinetic relations of the concentration–concentration type

$$1 + \frac{e}{g} = \varphi_\mathrm{G} + \varphi_\mathrm{E} \tag{9}$$

$$\varphi_\mathrm{G} + \varphi_\mathrm{D} + \varphi_\mathrm{M} + \varphi_\mathrm{A} = 1. \tag{10}$$

Integration of the differential kinetic equations (*7*) and (*8*) gives

$$\varphi_\mathrm{G} = \frac{1 + \dfrac{e}{g}}{1 + \dfrac{e}{g}\exp(kgt)} \tag{11}$$

$$\varphi_\mathrm{E} = \left(1 + \frac{e}{g}\right)\frac{\dfrac{e}{g}\exp(kgt)}{1 + \dfrac{e}{g}\exp(kgt)} \tag{12}$$

$$\varphi_\mathrm{D} = \frac{k_\mathrm{D}}{k}\,\frac{e}{g}\,\frac{\exp(kgt) - 1}{1 + \dfrac{e}{g}\exp(kgt)} \tag{13}$$

where $k = k_\mathrm{D} + k_\mathrm{M} + k_\mathrm{A}$.

These relations show the sigmoidal course with the inflexion point. After introduction of parameters P_1, P_2 and P_3, which are optimized by nonlinear regression, Eqs (*11*) and (*13*) acquire the forms

$$\varphi_\mathrm{G} = \frac{1 + P_1}{1 + P_1\,\exp(tP_2)} \tag{14}$$

$$\varphi_D = P_1 P_3 \frac{\exp(tP_2) - 1}{1 + P_1 \exp(tP_2)} \quad (15)$$

where $P_1 = e/g < 1$, $P_2 = kg$ and $P_3 = k_D/k$.

For the inflexion point, we may write

$$\varphi_{G\,inf} = \left(1 + \frac{e}{g}\right) / 2 \quad (16)$$

and its value must be smaller than 1, which is possible only when $e/g < 1$.

For $t \to \infty$ it could be written

$$\varphi_{G\,\infty} = 0 \quad (17a) \qquad \varphi_{E\,\infty} = 1 + \frac{e}{g} > 1 \quad (17b) \qquad \varphi_{D\,\infty} = \frac{k_D}{k} < 1 \quad (17c).$$

This means that at the end of the reaction, G is spent completely, the concentration of E increases and D is formed in the relative concentration smaller than 1.

EXPERIMENTAL

Chemicals

G. oxydans CCM 1783 (Czech collection of microorganisms, Brno, Czech Republic) immobilized in LentiCats® capsules from poly(vinyl alcohol) (LentiKat's Biotechnologies a.s., Praha, Czech Republic), glycerol p.a., potassium hydroxide p.a. 85% w/w, sulfuric acid p.a. 98% w/w, ethanol denaturated (all Lachema Brno, Czech Republic), yeast extract FermTech® (Merck, Darmstadt, Germany), defoamer Antispumin WA (Radka GmbH, Pardubice, Czech Republic), Persteril (32–36% peracetic acid, 5–12% hydrogen peroxide, 25% acetic acid, <1% sulfuric acid, Peroxides, Sokolov, Czech Republic).

Apparatus and Methods

All fermentative reactions were carried out in the apparatus depicted in Fig. 1. Every starting solution used for biotransformation included a mixture of three components: G, yeast extract (YE) and water. The defoamer Antispumin-WA was added to all starting solutions to remove the biological foam.

Under sterile conditions[15], 500 ml of aqueous solution containing 58.4 (±0.6) g l^{-1} G and 2.1 (±0.2) g l^{-1} YE were introduced into the reactor at a chosen temperature. The pH was adjusted and then held at 5.1. The flow rate of air was 10 l h^{-1} with the overpressure ca. 25 Torr. The reaction mixture was agitated with a propeller at 400 rpm. The biotransformation was started by the addition of a suitable amount of settled encapsulates with immobilized *G. oxydans* (see below). The pH decrease due to production of A was continually compensated by addition of aqueous KOH. In the liquid samples (ca. 5 ml) taken from the reaction

mixture in suitable time intervals, the reaction was stopped by acidification with 0.1 M HCl to pH 2 and the small amount of solid *G. oxydans* released from the pellets was removed by centrifugation. The concentrations of G and D in the samples were determined simultaneously by HPLC isocratic analysis.

Analytical Methods

HPLC isocratic determination of glycerol and dihydroxyacetone. HA pump LCP 4000 (Ecom Praha, Czech Republic), refractometric detector RIDK 102 (Laboratorní přístroje Praha, Czech Republic), ionex column Watrex IEX in H-form 250 × 8 mm, mobile phase 0.01 M H_2SO_4, flow rate 0.3 ml min^{-1}, sample dose 3 µl. The retention times of maxima of G and D peaks were 23 and 23.9 min, respectively. The concentrations of G and D were determined using calibration curves – area vs concentration with help of the PC program CSW 32.

Estimation of biomass content in capsules. Capsules with immobilized *G. oxydans* were thin lentils ca. 5 mm in diameter. A suspension of the capsules in aqueous 50% (w/w) solution of G (at this concentration the bioreaction is inhibited) was kept at 5 °C. A suitable amount of capsules was washed with water. The water remaining on the capsules was partly removed by drying between sheets of filter paper. Complete drying of capsules is not appropriate due to irreversible changes in the PVA structure. PVA coating of 2–5 g of partly dried capsules (moist capsules) was dissolved in 40 ml of hot water, the suspension of free *G. oxydans* was centrifuged in tubes (2.5 × 10 cm at 6000 rpm) and washed three times with 30 ml of water. The sediment was dried in a Petri dish at 105 °C for 3 h. From the mass of the dry sediment and initial mass of moist capsules, concentration of microorganisms (mg of dry biomass *G. oxydans* per 1 g of moist capsules) was calculated. The ratio was used for calculation of the initial amount of moist capsules representing the chosen initial *G. oxydans* concentration l (in g l^{-1}) in the reaction mixture. Temperature, pH and concentration of dissolved oxygen in the reaction mixture were continuously measured with a combined sensor.

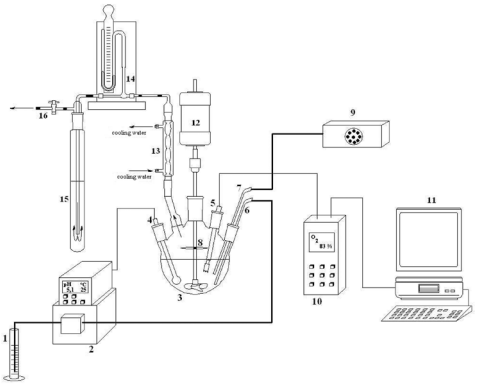

FIG. 1

1 stock KOH aqueous solution, 2 pH-stat, 3 fermentation batch reactor, 4 glass electrode, 5 oxygen electrode, 6 dosing of KOH solution, 7 glass tube with frit, 8 defoamer, 9 compressor, 10 multimeter, 11 PC, 12 electric propeller, 13 reflux condenser, 14 manometer, 15 manostat with silicone oil, 16 output from manostat

Note: Because of uncertain content of water in moist capsules and low weight of dry microorganisms in the dry *G. oxydans* sediment, this method gives rather an estimate than the exact biomass content in capsules.

RESULTS AND DISCUSSION

The fit of experimental and theoretical dependences was checked by their correlation coefficients (never smaller than 0.988) and visually (see Figs 2, 3, 5 and 7 below). As quantitative parameters, optimum values of rate constants k and k_D were obtained.

Kinetics of Biotransformation

The catalytic model is most simple and corresponds well with the physical idea of the reaction. However, the theoretical equations concentration vs time have not the experimentally found sigmoidal character and cannot therefore simulate the experiment.

Verification of the autocatalytic model was based on eight experiments performed in the batch reactor described in Fig. 1. The obtained experimental time dependences of φ_G and φ_D were evaluated according to Eqs (*14*) and (*15*) by nonlinear regression with software Origin 7.0. The total kinetic results obtained from these experiments are demonstrated in Table I. An

TABLE I
Kinetic results for the autocatalytic model. R_G^2 and R_{DHA}^2 are the determination coefficients. Their best value is 1

Experiment	$e/g \times 10^3$	$k \times 10^6$ (l g^{-1} s^{-1})	$k_D \times 10^7$ (l g^{-1} s^{-1})	R_G^2	R_{DHA}^2
EB1	3.6	1.32	7.54	0.99878	0.99844
EB2	1.94	1.18	6.57	0.99987	0.99928
EB3	2.84	1.29	6.73	0.99989	0.99528
EB4	3.97	1.04	6.19	0.99977	0.99952
EB5	4.20	1.26	7.17	0.99995	0.99772
EB6	4.71	1.13	7.06	0.99991	0.99868
EB7	3.78	1.57	8.75	0.99990	0.99954
EB8	4.64	1.10	6.47	0.99989	0.99902

example of consistency of dependences from experiment EB1 and their simulation by theoretical relations (*14*) and (*15*) is shown in Fig. 2.

The values of average determination coefficients in Table I, R_G = 0.9997 and R_D = 0.9984 (i.e. correlation coefficients 0.9998 and 0.9992), and also the graphical agreement of experimental and theoretical data in all 8 experiments confirm the validity of the purely statistical autocatalytic model. This model includes (see Eq. (*17*)) also the growth of microorganisms during the reaction. This growth is confirmed by the increasing initial ratios *e*/*g* in Table I in experiments EB1–EB8, which were performed with the same amount of poly(vinyl alcohol) pellets including increasing amount of *G. oxydans*.

Effect of Reaction Conditions

Influence of Initial Yeast Extract Concentration

The time dependences of *G* and *D* for four initial concentrations of sterilized aqueous YE ([YE] = 1, 2.5, 4 and 5 g l^{-1}) were measured. Temperature 25 °C, initial concentrations of G (*g* = 58.6–58.8 g l^{-1}) and of biomass (*l* = 3.78 g l^{-1}) were kept constant for each experiment. The obtained curves φ_G and φ_D vs *t* are presented in Fig. 3. Parameters P_2 and P_3 were found by nonlinear regression and from these parameters the values of *k* and k_D were

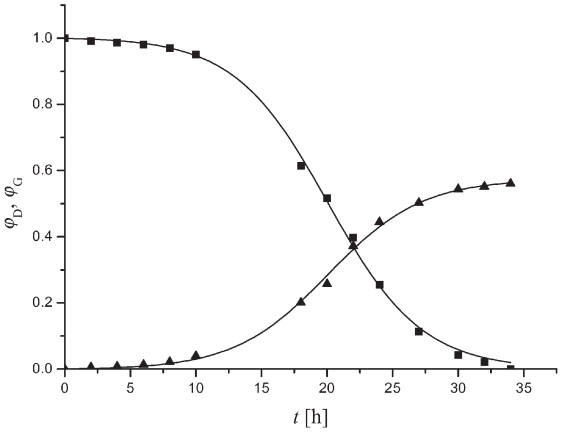

FIG. 2
Autocatalytic model, experiment EB1. Simulation (full lines) of experimental dependences (points) φ_G (■) and φ_D (▲) vs time (*t*) according to Eqs (*14*) and (*15*)

calculated (Table II). The dependence of rate constants k and k_D on concentration of YE is demonstrated in Fig. 4.

With increasing YE concentration (up to ca. 3.5 g l^{-1}), the rates of biotransformation reaction (k) and D production (k_D) increase. For higher YE concentrations, both rate constants sharply decrease. The presence of YE in the bioreaction is necessary. In the absence of nutrients from YE, no enzyme responsible for biotransformation of G to D is produced. On the contrary, at too high YE concentrations, the rate of D production rapidly falls. This can be caused, e.g., by the reduced permeability of capsules to reaction components. In the same bioconversion with free cells, the maxi-

TABLE II
Effect of initial concentration of YE on kinetic parameters k, k_D

Parameter $l\ g^{-1}\ h^{-1}$	$[YE]_0$, g l^{-1}			
	1	2.5	4	5
k	0.00462	0.005343	0.005339	0.00386
k_D	0.00323	0.003572	0.00373	0.002587

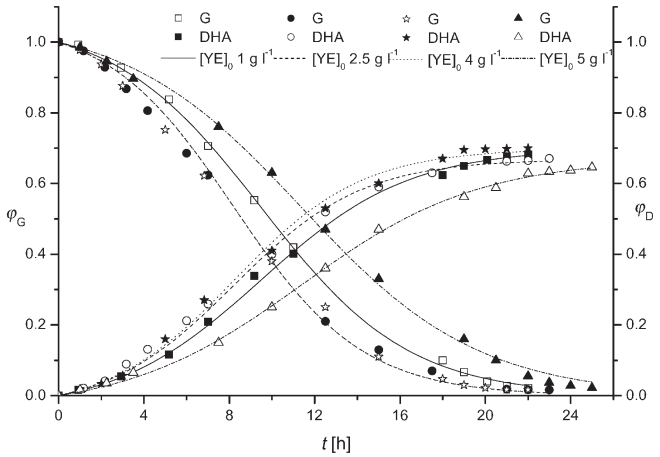

FIG. 3
Dependences of relative mass concentration of G and DHA (φ_G and φ_D) vs time (t) for four initial concentrations of yeast extract $[YE]_0$. Points, experiment; lines, simulations according to Eqs (*14*) and (*15*)

mum $[YE]_0$ = 5 g l^{-1} used in this work was dosed routinely without any inhibition effect.

Influence of Initial Concentration of Glycerol

The time dependences of *G* and *D* for four initial concentration of glycerol (*g* = 20.6, 58.6, 110.8 and 219.8 g l^{-1}) were measured. Temperature 25 °C, initial concentration of YE (2.5 g l^{-1}) and of biomass (*l* = 4.3 g l^{-1}) were kept constant in each experiment. The resulting curves of φ_G and φ_D vs *t* are shown in Fig. 5. Parameters *k* and k_D are presented in Table III. The dependence of rate constants on initial concentration of glycerol is given in Fig. 6.

TABLE III
Effect of initial concentration of G on kinetic parameters *k*, k_D

Parameter l g^{-1} h^{-1}	*g*, g l^{-1}			
	20.6	58.6	110.8	219.8
k	0.01436	0.00510	0.00114	0.00224
k_D	0.00862	0.003421	0.00064	0.00075

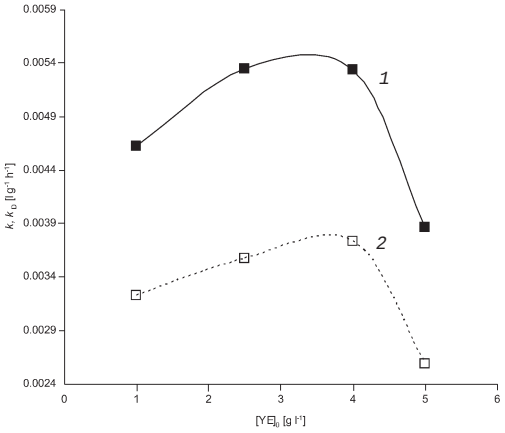

FIG. 4
Effect of initial yeast extract concentration, $[YE]_0$, on the rate constants *k* (*1*) and k_D (*2*). Points, experiment; see Table II

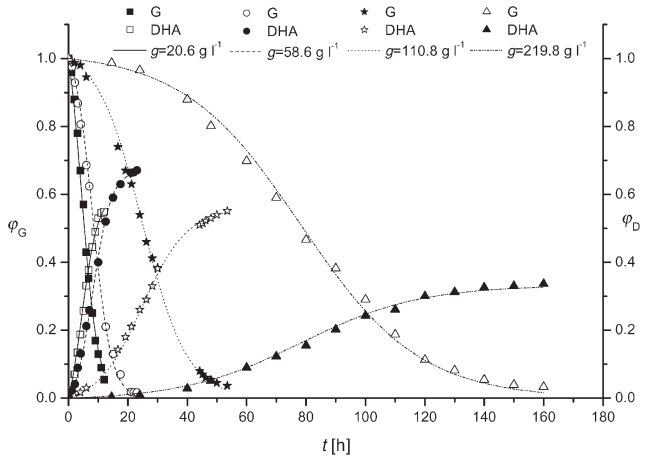

FIG. 5

Dependences of relative concentration of G and DHA (φ_G and φ_D) vs time (t) for four initial concentrations of glycerol g. Points, experiment; lines, simulations according to Eqs (*14*) and (*15*)

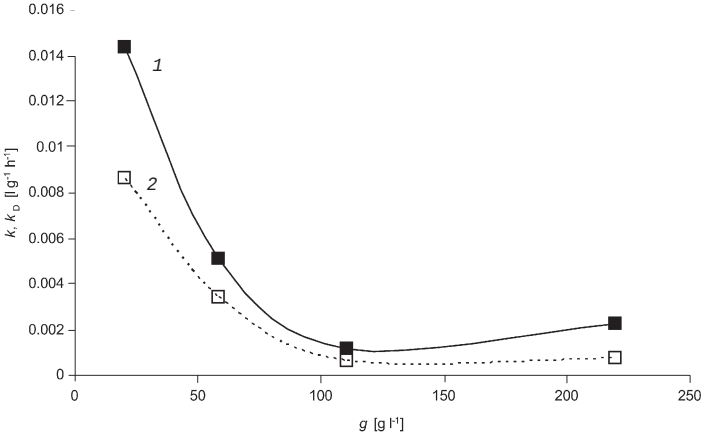

FIG. 6

Effect of initial glycerol concentration g on the rate constants k (*1*) and k_D (*2*). Points, experiment; see Table III

The rate of D production decreases with increasing initial concentration of G. At initial concentration g = 20.6 g l^{-1}, the reaction was complete (G = 0) after 12 h. At g = 219.8 g l^{-1} this time period was fifteen times longer. As we can see from Figs 5 and 6, at g > 100 g l^{-1} the reaction time increased and the k_D value markedly decreased. On the other hand, a very high value of g increased the overall biotransformation rate, giving probably other products than D. The initial concentration of G over 100 g l^{-1} is therefore disadvantageous.

Influence of Reaction Temperature

The time dependencies of G and D for three reaction temperatures (20, 25 and 30 °C) were measured. The initial concentration of YE (2.5 g l^{-1}), initial concentrations of biomass (l = 4.12 g l^{-1}) and of glycerol (g = 58.6 g l^{-1}) were kept constant in each experiment. The obtained curves φ_G and φ_D vs t are presented in Fig. 7. Parameters k and k_D are given in Table IV. The dependences of the rate constants on reaction temperature are presented in Fig. 8.

The dependences of biotransformation rate constants on temperature in Fig. 6 show that the optimal reaction temperature lies between 25 and 30 °C. In this interval the difference in the rate constants is small. The lower solu-

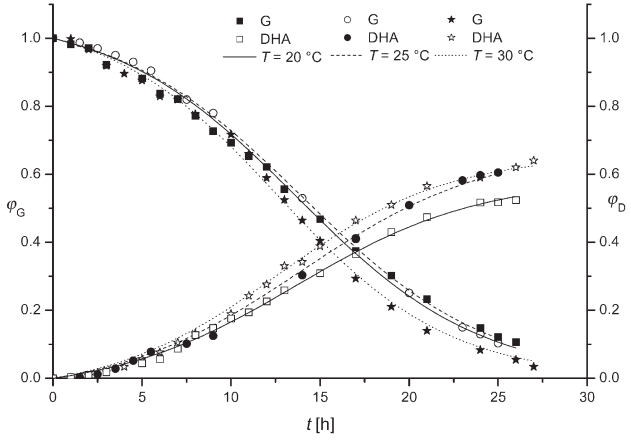

FIG. 7
Dependences of relative concentration of G and DHA (φ_G and φ_D) vs time (t) at three various temperatures. Points, experiment; lines, simulations according to Eqs (*14*) and (*15*)

bility of oxygen in the reaction mixture at 30 °C had to be compensated by more intensive aeration. Temperatures over 30 °C could not be studied due to the solubility of PVA capsules in water. At 15 °C, the biotransformation practically did not even start after 10 h, i.e. no perceptible decrease in G and increase in D could be measured.

TABLE IV
Effect of temperature on kinetic parameters k, k_D

Parameter $l\ g^{-1}\ h^{-1}$	Temperature, °C		
	20	25	30
k	0.00311	0.00303	0.00334
k_D	0.00182	0.00206	0.00220

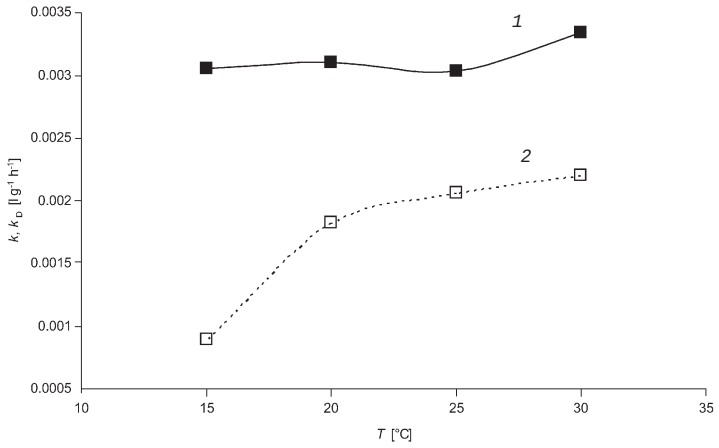

FIG. 8
Effect of temperature on the rate constants k (*1*) and k_D (*2*). Points, experiment; see Table IV

Conclusions

Kinetics of glycerol biotransformations into 1,3-dihydroxyacetone by *G. oxydans* immobilized in poly(vinyl alcohol) pellets was at 25 °C successfully described by the formal autocatalytic model consisting of three parallel steps which produce, besides dihydroxyacetones, also glyceric acid and consume a part of glycerol for the growth of the mentioned microorganism. The rate constant of dihydroxyacetone production step k_D and the sum of rate constants k of all three steps of this model were calculated. The determined values of rate constants can be used to calculate the optimal concentrations of G and D in the production of 1,3-dihydroxyacetone by *G. oxydans* encapsulated in poly(vinyl alcohol) gel.

The effect of initial yeast extract and glycerol concentrations and reaction temperature on the course of this biotransformation was studied. The proposed three-step kinetic model of the biotransformation was experimentally verified also for the temperature interval 20–30 °C. Increasing initial concentration of yeast extract (up to 3.5 g l^{-1}) increases and of glycerol markedly decreases the rate of production of 1,3-dihydroxyacetone. At temperatures ≤15 °C, no 1,3-dihydroxyacetone is produced, temperatures over 30 °C already dissolve the PVA capsules in water. In the range of 25–30 °C, the rate of the 1,3-dihydroxyacetone production is practically constant.

Optimal conditions of the studied bioconversion are: reaction temperature 25–30 °C, initial yeast concentration 2–4 g l^{-1} and initial concentration of glycerol 20–50 g l^{-1}.

SYMBOLS

A	glyceric acid
D, *D*	1,3-dihydroxyacetone, mass concentration of D (g l^{-1})
E	enzyme(s) in *G. oxydans*
G, *G*	glycerol, mass concentration of G (g l^{-1})
g, *e*	initial mass concentrations of G, E (g l^{-1})
k_i	rate constant of *i*-th reaction (l g^{-1} h^{-1})
$k = k_D + k_M + k_A$	
$[L]_0$	initial molar concentration of *G. oxydans* (mol l^{-1})
l	initial mass concentrations of dry *G. oxydans* (g l^{-1})
M	products of metabolic processes
PVA	poly(vinyl alcohol)
YE, $[YE]_0$	yeast extract, initial mass concentration of YE (g l^{-1})
φ_G, φ_D	relative (dimensionless) concentrations *G*/*g*, *D*/*g*

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