Applicability of Mathematical Models in Defining the Behaviour Kinetics Distinction Among Microbial Strains

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Mathematical models were applied to define the behaviour kinetics distinction among microbial strains. In the first series of experiments the growth kinetics of microbial colonies of several *S. rimosus* mutant strains cultivated on agar plates were compared. Then, the interest was focused on the chosen two strains, in order to express mathematically their differences with respect to their colony growth and antibiotic biosynthesis kinetics. Finally, the behaviour of selected three *S. rimosus* derivative strains at different culture conditions was subjected to the study, with an aim to define strain distinction parameters. Mathematical models based on the three-dimensional growth concept and describing the microorganism growth, substrate uptake and antibiotic biosynthesis kinetics were developed. The computer simulation was applied to verify the applicability of mathematical models. The excellent agreement of computer simulation with experimental data confirmed the hypothesis that the kinetics parameters can be successfully applied to define the behaviour distinction among different *S. rimosus* strains. In the case of selected three strains, *S. rimosus* R6–500, *S. rimosus* MV9R-1 and MV9R-2, it was established that they can be distinguished by their growth kinetics parameters, their substrate uptake kinetics parameters and their antibiotic biosynthesis kinetics parameters.The strain *S. rimosus* R6–500 showed to be superior with respect to all kinetics parameters, the strain *S. rimosus* MV9R-2 showed to be slightly inferior to it, whereas the strain *S. rimosus* MV9R-1 showed to be inferior with respect to the both mentioned strains, especially because it showed the pronounced active biomass reduction rate at all investigated culture conditions. Based on these and the corresponding previous results one can conclude that appropriate mathematical models can be recommended for defining parameters of microbial behaviour distinction among different microbial strains of *S. rimosus* species.

Key words:
Microbial kinetics, mathematical models, *Streptomyces rimosus,* microbial strain distinction, growth, substrate uptake, product formation, morphology

Introduction

Generally, the behaviour of any microorganism is determined by its genetic properties and depends on its culture history and its actual cultivation conditions. For a behaviour one can consider that it is a manifestation of enormous number of different events, which happen in microbial cells and their close environment due to their activities and which can be more or less interconnected, spontaneous or induced. Depending on their characteristics, the mentioned events can be systematically grouped to facilitate their studies and studies of their consequences. Commonly, events refer mainly to biochemical reactions and physical movements and therefore they can be grouped in accordance to their similarity and roles in cell growth, substrate uptake, product formation, energy evolution, cell differentiation and agglomeration, cell death and autolysis, cell and culture morphology, effects on culture environment, *etc*. Although one can focus on the study of any particular event regardless of its nature, usual practice is a simple approach to the study of process kinetics, *i.e.* to study the growth kinetics, substrate uptake and product formation kinetics, mass and energy transfer phenomena, effects of different factors on process kinetics, *etc.* It is known that kinetics of any process event can be ex-

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pressed, at least approximately, by means of a corresponding mathematical model. Since genetic properties of any microorganism mainly determine its behaviour, it follows out that process kinetics of any microbial culture is dependent on genetic properties of particular members of a microbial culture. In the most simplest cases of microbial cultures only one microorganism with strictly defined genetic properties is present. If a microbial culture is started by introducing an inoculum of pure microbial culture with stable genetic properties, then one can expect the pure microbial population to be propagated. Since culture conditions influence properties of microbial cells, microbial cultures with cells differing in their properties result when culture conditions change during the cultivation, as commonly happens in batch cultures. However, one can expect phenotype changes of cell properties to happen preferentially and with high probability, whereas genotype changes can be expected with extremely low probability. When discussing the problem of appearance of genotype changes in cell properties it should be pointed out that the appearance of cells with changed genetic properties becomes important if one tends to isolate microorganisms with better properties, and especially if one should prevent a "culture degeneration", *i.e.* the predomination of the "wild" strain over the "normal" strain.

Frequently, the aim is to discover the strains with desired improved or new properties with reference to known, exploited strains. Then the problem of how to recognise new strains usually appears. Therefore, regardless of whether the study refers to the process kinetics of microbial cultures, or to the discovery of new microbial strains, it becomes important to define characteristics of microbial strains in such a way that one can predict their behaviour during their cultivation. Since microbial culture kinetics can be well described by appropriate mathematical models, one can consider that the mathematical models can be applied to define the behaviour kinetics distinction among microbial strains. Because antibiotics are still of high importance for existence of modern human society, the idea to define the behaviour kinetics distinction among antibiotic producing microbial strains appeared to be very attractive. It was supposed that the experiments referring to oxytetracycline biosynthesis with different *Streptomyces rimosus* derivative strains could be an excellent example to demonstrate the validity of the above statement. In the first series of experiments the biosynthetic activities and the growth kinetics of microbial colonies of several *S. rimosus* mutant strains cultivated on agar plates were compared. Then, the interest was focused towards the chosen two strains, in order to express mathematically their differences with respect to their colony growth and antibiotic biosynthesis kinetics[1]. Mathematical models based on the three-dimensional growth concept were successfully applied in describing process kinetics referring to the production of different products by microbial cultures of different mycelial microorganisms. Such a result has been used in present study to investigate the behaviour of selected three *S. rimosus* derivative strains at different culture conditions, with an aim to define strain distinction parameters.

Development of appropriate mathematical models

When the aim of mathematical modelling is a mathematical description of fundamental behaviour kinetic characteristics of particular microbial strains, then at the start of study it becomes sufficient to describe the behaviour of mentioned strains in their batch cultures, *i.e.* it becomes sufficient to define process kinetics parameters specifically for each of investigated strains, only on the basis of experimental data from corresponding batch cultures, where one can include agar plates with microbial colonies as well. Therefore, one can focus here only on the consideration of batch culture process kinetics and development of mathematical models, which can adequately explain experimental batch culture data. Since the purpose of present work is to define mathematically specific kinetic characteristics of mycelial microorganisms, *i.e.* of *S. rimosus* derivative strains, it was decided to investigate applicability of the mathematical models developed on the basis of a three-dimensional growth concept. The support for such a decision certainly can be found in the series of already published data referring to the description of growth kinetics of different mycelial microorganisms[1–13] as well as to the description of kinetics of substrate uptake[8–11,13] and product formation[1,3,4,6–13] in cultures of mycelial microorganisms, namely of *streptomycetes*[1–5,7–10,12,13] and *aspergilli*[1,6,11,13]. By way, it is important to point out that the applicability of the three-dimensional growth concept was tested in cultivating microorganisms as microbial colonies[1] as well as in submerse batch cultures[2–13], fed batch cultures[4, 6–11, 13] and repeated fed batch cultures[4,6,7,9].

Based on published data the equation

$$d\gamma_x/dt = k_1 \cdot \gamma_x^{2/3} - k_2 \cdot \gamma_x \qquad (1)$$

and its extended form

$$d\gamma_x/dt = k_1 \cdot \gamma_x^{2/3} - k_2 \cdot \gamma_x - k_3 \cdot \gamma_x \cdot t \qquad (1a)$$

can be generally recommended as fundamental ones to describe growth kinetics of mycelial microbial

strains in their batch cultures, if a long period after lag phase and the first transient phase is considered. However, as demonstrated recently,[13] eq. 1 can be modified in such a way that the resulting eq. 2 can explain growth kinetics even when the lag and transient periods are included into consideration. Then

$$d\gamma_x/dt = \Phi_x \cdot k_1 \cdot \gamma_x^{2/3} - k_2 \cdot \gamma_x \quad (2)$$

if the physiological factor of biomass adaptation, Φ_x, is defined as follows

$$\Phi_x = \gamma_x/(K_x + \gamma_x) \quad (3)$$

Eq. 1 can be modified for a description of growth kinetics of microbial colonies. Then changes of colony diameter are commonly considered during the growth of colonies, and it was found [1] that the equation

$$dD_c/dt = k_{1D} - k_{2D} \cdot D_c \quad (4)$$

explained well the growth kinetics of colonies of two different *S. rimosus* R6 derivative strains. The fact that the eq. 1 was successfully applied in explaining the growth kinetics of tumour spheroids[14] with reference to tumour spheroid diameters and volumes as well as to the number of viable tumour spheroid cells, leads to propose the following expression, defining the relation between the maximal (D_m), initial (D_0) and actual (D) tumour spheroid diameters, for colonies of *S. rimosus* R6 derivative strains:

$$3 \ln[(D_m - D_0)/(D_m - D)] = k_2 \quad (5)$$

Substrate uptake kinetics can be simply expressed by equation

$$d\gamma_s/dt = -q_s \cdot \gamma_x \quad (6)$$

However, it was observed that the specific substrate uptake rate, or metabolic quotient of substrate uptake (q_s), depends on substrate concentration and can be affected by product concentration.[8,13] Therefore, the equation

$$d\gamma_s/dt = -q_{sm} \cdot \Phi_s \cdot \gamma_x \quad (7)$$

where

$$\Phi_s = \gamma_s/(\alpha_i \cdot K_s + \gamma_s) = \gamma_s/[(1 + f_{ip} \cdot \gamma_p) \cdot K_s + \gamma_s] \quad (8)$$

can be proposed to express kinetics of consumption of some substrates (*e.g.* of carbohydrates).

Since inorganic phosphate in fermentation broth can influence growth and product formation,[8,15,16] kinetics of its uptake should also be considered. One of possibilities is to apply the equation

$$d\gamma_{sf}/dt = -q_{sf} \cdot [\gamma_{sf}/(K_f + \gamma_{sf})] \cdot \gamma_x \quad (9)$$

Different products can be formed and their formation kinetics is depedent on different factors. To express the kinetics of oxyteracycline formation a general equation

$$d\gamma_p/dt = q_p \cdot \gamma_x \quad (10)$$

can be proposed. Because it is known that carbon source[8,13,17] and inorganic phosphate[8,15,16] concentrations influence oxytetracycline biosynthesis rate, a more adequate equation is suggested, *i.e.*

$$d\gamma_p/dt = q_{pm} \cdot (1 - \gamma_{sf}/\gamma_{sfm}) \cdot \Phi_s \cdot \gamma_x \quad (11)$$

If oxytetracycline biosynthesis refers to the process of oxytetracycline production by microbial colonies, then instead of eq. 10 the following equation

$$dP/dt = Q_P \cdot X \quad (12)$$

can be applied to express the rate of oxytetracycline mass production by particular colony. Due to the produced antibiotic, the corresponding inhibition zone can be described by the equation[1]

$$dD_z/dt = Q_{PX} \cdot X/D_z \quad (13)$$

For antibiotic producing biomass proportional to the second power of its colony diameter (Fraction of colony biomass volume capable to produce antibiotic depends on colony morphology and changes with colony age. Spheroidal character of colonies is more pronounced in younger colonies and decreases with colony age.[1] It was established[18] that with decreasing the designed distances between colonies and with increasing the number of colonies in a particular colony vicinity the maximal values of colony diameters become correspondingly reduced), relation (13) becomes

$$dD_z/dt = Q_{D2} \cdot D_c^2/D_z \quad (14)$$

whereas for such biomass proportional to the third power of its colony diameter it follows that

$$dD_z/dt = Q_{D3} \cdot D_c^3/D_z \quad (15)$$

It is generally considered that in eqs. 13–15, $D_z \geq D_c$ because the minimal D_z refers to the diameter of surface area occupied by colony biomass, where sensitive test-microorganism cannot grow regardless of whether microbial colony is capable to produce antibiotic or not. By application of common methods of assaying inhibition zones produced by microbial colonies it is not possible to estimate antibiotic quantities equivalent to inhibition zones lower than the surface area occupied by investigated microbial colony. Recently, this problem was solved adequately by applying the method where agarised media layers were fortified with inert me-

tallic nets.[19,20,21] Therefore, it becomes possible to extend the range of mathematical model applicability by modifying the denominator in eqs. 13–15. Since the inhibitory effect of antibiotic can be expressed only if antibiotic is present, *i.e.* after being produced by microbial colony, one of possibilities for denominator substitution appears to be by application of the expression

$$D_z = 1 + D_{za} \tag{16}$$

where D_{za} refers to the inhibition zone diameter determined by the proposed new method.

The potency index (I_p) can be used as one of the criteria for selecting strains of better antibiotic productivity,[22] and it is defined as the ratio of product formation zone (inhibition zone) diameter against colony diameter, *i.e.*

$$I_p = D_z/D_c \tag{17}$$

If the mutant strain biomass, due to some reasons, reduces its ability to consume substrate and to form antibiotic, then an adequate modification of the mathematical model should be accepted. If one supposes that only a part of biomass conserves its stable activity with reference to substrate uptake and antibiotic synthesis (active biomass, X_{ac}), one of the possibilities to express the rate of substrate uptake is by equation

$$d\gamma_s/dt = -q_{sm} \cdot \Phi_s \cdot \gamma_{xac} \tag{18}$$

where for changes of active biomass concentration (γ_{xac}) the following equations have been proposed:

$$d\gamma_{xac}/dt = d\gamma_x/dt - d\gamma_{xin}/dt \tag{19}$$

$$d\gamma_{xin}/dt = k_4 \cdot \gamma_x \cdot \gamma_p - k_5 \cdot \gamma_{xin} \tag{20}$$

Then for expression of antibiotic formation kinetics the equation

$$d\gamma_p/dt = q_{pm} \cdot \Phi_s \cdot \gamma_{xac} \tag{21}$$

can be proposed. The effect of inorganic phosphate on antibiotic synthesis in such a case can be neglected.

If aerated and agitated biochemical reactors are used to perform the process of antibiotic production, the oxygen transfer and uptake should commonly be considered. Starting from theoretical considerations and by using experimental experience, following equation

$$d\gamma_{OL}/dt = k_L a \cdot (1 - f_{kLa} \cdot \gamma_x) \cdot (\gamma_{OL}^* - \gamma_{OL}) - q_{O_2} \cdot \gamma_x \cdot [\gamma_{OL}/(K_{OL} + \gamma_{OL})] \cdot \{\gamma_s/[(1 + f_{ip} \cdot \gamma_p) \cdot K_s + \gamma_s]\} \tag{22}$$

can be proposed to express, at least roughly, the kinetics of changes of dissolved oxygen concentration during the process. One can expect that different microbial strains could also be distinguished on the basis of their parameters referring to the kinetics of oxygen uptake.

Materials and methods

Microorganisms

In the first and the second series of experiments the derivatives of *S. rimosus* R6 have been applied. After mutagenic treatment with *N*-methyl-*N*-nitrosoguanidine, five morphologically different colonies have been isolated and then designated as variants 1001, 1002, 1004 and 1005. Bioassays for produced antibiotic have been performed by *S. rimosus* R6–615, a non-producing mutant with limited resistance to oxytetracycline (OTC)[1]. In the third series of experiments, three selected strains appearing spontaneously in the *S. rimosus* R6–500 culture,[23] nominated as *S. rimosus* R6–500, *S. rimosus* MV9R-1 and *S. rimosus* MV9R-2[24,25] have been applied.

Culture media

A: Agar media for colony growth: a) Malt agar medium (MA) containing [g L^{-1}]: malt extract, 4; glucose, 4; agar, 20 (al "Difco", Detroit, USA); b) Maltose – CSL medium (MC) prepared from the filtrate of the medium of following composition [g L^{-1}]: maltose (Difco), 70; dry CSL, 9; $CaCO_3$, 6; $(NH_4)_2SO_4$, 3.5; NH_4Cl, 3; $MgSO_4 \cdot 7H_2O$, 1; $MnSO4 \cdot 7H_2O$, 0.1; $CoCl_2.6H_2O$, 0.005; Upon filtration agar (Difco) has been added. The test organism was applied in molten MC containing less of agar (6 g L^{-1}) (*1*); c) MCYE medium, *i.e.* MC medium with yeast extract (4 g L^{-1}); d) MAYE medium containing [g L^{-1}]: glucose, 4; malt extract, 10; yeast extract, 4; agar, 4; e) Soya-manitol-agar medium (SM) containing [g L^{-1}]: soybean meal, 20; manitol, 20; agar, 20;

B: Media for submerse cultures of *S. rimosus* strains: a) Medium for inocula preparation (MIC) containing [g L^{-1}]: dextrin, 40; CSL (50 %), 16; $CaCO_3$, 7; $(NH_4)_2SO_4$, 2; lactic acid, 1.4 mL; b) Medium for oxytracycline production (MOP) containing [g L^{-1}]: dextrin, 100; $CaCO_3$, 10; CSL (50 %), 15; $(NH_4)_2SO_4$, 7; NH_4Cl, 2.5; $MgSO_4 \cdot 7H_2O$, 2; $MnSO_4 \cdot 7H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.006; NaF, 0.0001; $ZnSO_4 \cdot 7H_2O$, 0.02; unidan (antifoam agent), 0.3 mL; BAN (α – amylase), 0.04;

Remark: Prior to use or storage, all media were sterilised applying adequate heat sterilisation methods.

Preparation of single colonies

Well-separated colonies of chosen isolates have been used. Colonies have been stabbed with a sharp needle (or glass rod) in order to plate their material onto medium surface[1,19,20]. Agar plates of uniform medium thickness of 3 mm (30 mL of medium in 110 mm Petri dish), with 6 colonies growing at an equal distance from each other, plate's rim and the centre, as well as the plates with only one colony growing in the plate centre have been applied.

Colony size evaluation

At defined time intervals, using the transparent millimetre paper (graph paper), the colony diameter (average) and surface area were estimated. The chosen colonies were then carefully separated from the medium in order to be used for their α,ε-L,L-diamino pimelic acid (DAPA) estimation. Hydrolysis with 6 mol L^{-1} HCl at 105 °C for 20 h and thin layer chromatography[3,26] have been applied for DAPA mass determination.

Evaluation of OTC produced by colonies

Plates containing single colonies were overlayed with 7 mL of MA with 0.6 % of agar, containing about $5 \cdot 10$ C.F.U. of the test strain, and then incubated at 32°C overnight. Then the inhibition zones were recorded and agar discs of 6 mm in diameter were cut out with a cork borer from the colony centre along the diameter of the inhibition zone. Both disks with known OTC concentrations and those cut from inhibition zones were transferred onto large dishes ($300 \cdot 300$ mm) containing solidified MA medium seeded with the test strain. After one overnight incubation the inhibition zone diameters were measured. Amounts of OTC produced by single colonies were estimated by applying the expression

$$P = \sum_{i=1}^{n} (S_i - S_{i-1}) \cdot p_i \qquad (23)$$

where $S_i = R_i^2 . \pi$, R_i = distance from the colony centre, p_i = mean OTC concentration in the inhibition zone ring between R_i and R_{i-1}, $R_0 = 0$.[1]

Submerse batch cultures

Submerse batch cultures were performed a) as shaken flask cultures and b) as batch cultures in laboratory bioreactors.

a) Shaken flask cultures: 7 Erlenmeyer (conical) flasks of 500 mL, each containing 50 mL of nutrient medium MOP were inoculated with 10 % of inoculum of investigated strain (Strains *S. rimosus* R6–500, *S. rimosus* MV9R-1 and *S. rimosus* MV9R-2 were investigated). Inoculated flasks were subjected to the cultivation process at 28 °C on rotary shaker for 6 d (144 h). Every day (24 h) one of flasks was used to analyse biomass, carbohydrate and oxytetracycline concentrations in its fermentation broth.

b) Batch cultures in laboratory bioreactors: Same strains and the same nutrient medium (MOP) as indicated under a) were applied. Cultivation was performed in laboratory bioreactors of 14 L ("Microferm MF-214", New Brunswick Sci., USA) filled with 8 L of medium. After inoculation, air supply of 1 L L^{-1} min^{-1} and agitation with stirrer rotation speed of n = 800 min^{-1} were applied. Water evaporated from the culture was compensated by periodic additions of adequate amounts of sterile water. Process course was followed up and samples of fermentation broth were analysed to estimate biomass, total carbohydrate and oxytetracycline concentrations in fermentation broths.

Determination of biomass, carbohydrate and oxytetracycline concentrations

Determination of biomass concentration: 10 mL of fermentation broth was put into centrifugation cuvette of 15 mL and then a centrifugation with n = 10 000 min^{-1} at 4 °C was applied. After effluent elimination, the sediment was re-suspended in demineralised water and 0.1 mL of α-amylase preparation and 0.05 mL of glucoamylase preparation were added to the suspension. Reaction mixture was incubated at 60 °C for 30 min in order to hydrolyse carbohydrates, which could affect dry biomass estimation. After incubation, concentrated HCl solution was added to adjust medium pH to 1.8, *i.e.* an acidity convenient for an efficient $CaCO_3$ degradation. $CaCO_3$ being degraded and dissolved (15 min), centrifugation for 15 min was applied again, effluent eliminated and then acetone added to re-suspend sediment. After centrifugation of suspension and effluent elimination, cuvette containing a wet biomass was exposed to drying for 2 hours at 60 °C in vacuum, and then at 105 °C. Dry biomass was estimated by weighting upon a storage in exicator.

Determination of carbohydrate concentration: Autoanalyser "Technicon TRAACS 800" (Brann und Luebbe GmbH, Norderstedt, D) was applied. Continuous hydrolysis of samples (8 min, 6 mol L^{-1} HCl at 99 °C) followed by dialysis against fericyanid was applied. Reduction of yellow $K_3Fe(CN)_6$ into colourless ferocyanid resulted due to the presence

of free sugars. One monitors colour intensity at 420 nm to estimate content of total carbohydrates expressed as starch.

Determination of OTC concentration: Spectrophotometric method of *Monastero* et al. [27] based on an absorbance at 354 nm, modified by *S. Gamu*lin and *M. Bošnjak* (internal procedure of PLIVA research laboratories) was applied. Fermentation broth sample was acidified by addition of concentrated HCl solution. Homogenised acidified sample of pH 1.8 was then filtered by using filter paper, and obtained filtrate was used for measurements. These were performed applying dilutions to appropriate concentration and in accordance to the corresponding solutions of the standard, *i.e.* of pure oxytetracycline.

Computer simulation

In the first phase of study the computer simulations were performed by using the computer "Hewlett Packard – 9845 B". Later, during the key phase of study, based on a previous successful application of computer simulations in the theoretical studies of some complex microbial culture systems,[28] the corresponding simulation programme suitable for the *MicroMath Scientist Ver. 2.0* software package was developed and series of computer simulations was performed applying PC compatible computer.

Results and discussion

Results demonstrating the applicability of mathematical models to experimental data are presented in Tables 1–4 and Figs. 1–10. Tables 1 and 2 refer to the first and the second series of experiments, whereas Figs. 1–10 and Tables 3 and 4 refer to the third series of experiments. Table 1 is selected as a part of data of the poster presented already in 1985 at the course of the NATO Advanced Study Institute on Recent Developments in Biotechnology, held March 17–29, 1985 in Troia, Portugal (*M. Bošnjak*, *J. Pigac*, *M. Vampola* and *M. Vešligaj*: Kinetics of oxytetracycline biosynthesis during the growth of colonies of oxytetracycline producing strains and the mathematical model, abstracts (prepared by LNETI, J. C. Duarte, ed.),distributed to participants)) and refers to an applicability of eq. 5 in explaining experimental colony diameters of morphologically different mutants of *S. rimosus* R6 strain. As shown in Table 1, data clearly confirmed the adequacy of eq. 5 in explaining experimental results and in defining the differences in the behaviour of different mutant strains with respect to their colony growth kinetics. Excellent correlation coefficients for four morphologically different isolates (isolates 1002 to 1005) and very good one for isolate 1001 were established, if adequate kinetic parameter values, *i.e.* k_2 and D_m values, were applied for particular isolates. Table 2 contains data showing differences between isolates 1001 and 1003 with reference to their colony growth and antibiotic synthesis kinetics, as well as to the agreement of theoretical with experimental data. The differences between two isolates are very pronounced with reference to both growth kinetics and product formation parameters, whereas the agreement of theoretical with experimental data was very good for growth kinetics data of both isolates and antibiotic formation kinetics of the isolate 1003. The correlation for antibiotic produced by colonies of the isolate 1001 was found to be slightly less adequate. It follows that presented data suggest the applied mathematical model represented by eqs. 1, 1a, 4, 13–15 can be accepted, as already was previously concluded, on the basis of same and additional data.[1]

Figs.1 and 2 refer to cultures of *S. rimosus* R6–500 and demonstrate cultivation process kinetics of this strain. Excellent agreement of theoretical with experimental data for applied mathematical model composed of mathematical eqs. 2, 3, 7–9 and 11 has been found, disregarding whether data refer to shaken flask cultures or to the batch culture in laboratory bioreactor. Since a trial to apply the same mathematical model for explaining experimental data referring to cultures of *S. rimosus* MV9R-1 was unsuccessful, it was necessary to modify the mentioned mathematical model. Experimental data and data of preliminary computer simulations suggested the use of mathematical model defined by eqs. 2, 3, 18–21. Computer simulation confirmed this hypothesis, as can be well seen in Figs. 3 and 4.

Based on a rough insight into experimental data it was expected for the strain *S. rimosus* MV9R-2 to show some similarity with respect to *S. rimosus* R6–500 behaviour. Therefore, an applicability of the same mathematical model in explaining the behaviour of both strains was expected. Data in Figs. 5 and 6 completely confirmed the expectation. Similarity between two strains with respect to their process kinetics is quite evident. However, data suggest the strain MV9R-2 appears to be inferior in comparison with *S. rimosus* R6–500 strain. An excellent way to demonstrate behaviour differences between investigated strains was by comparing their theoretical specific growth, substrate uptake and product formation rates, as shown in Fig. 7 a,b,c. Differences are better high-lighted if kinetics of substrate uptake and product formation are considered.

Table 1 – *Applicability of eq. 5 in describing growth kinetics of colonies of morphologically different isolates obtained after mutagenic treatment of S. rimosus R6*

Isolate name	k_2/d^{-1}	D_m/mm	Correlation coefficient, R
1001	0.360	16.0	0.98122
1002	0.350	13.0	0.99468
1003	0.360	24.0	0.99976
1004	0.103	30.0	0.99026
1005	0.220	30.0	0.99931

Table 2 – *Kinetics of oxytetracycline biosynthesis during the growth of microbial colonies. Fitting simulated to experimental data*

Strain	Biomass evaluated as	Equations	Parameter values*				Correlation coefficient
			k_{1c}	k_{2c}	k_{3c}	Q	r
1001	colony diameter	/4/	2.75	0.2	0.0	–	0.99985
1001	colony diameter.	/4/, /14/	2.75	0.2	0.0	2.0	0.97540
1001	colony diameter	/4/, /15/	2.75	0.2	0.0	0.2	0.97606
1001	DAPA	/1a(X)/	2.8	0.003	0.045	–	0.98472
1001	DAPA	/1a(X)/, /13/	2.8	0.003	0.045	0.85	0.96975
1003	colony diameter	/4/	3.62	0.15	0.0	–	0.99792
1003	colony diameter	/4/, /14/	3.62	0.15	0.0	0.66	0.99925
1003	colony diameter	/4/, /15/	3.62	0.15	0.0	0.046	0.99645
1003	DAPA	/1a(X)/	5.65	0.7	0.0085	–	0.99496
1003	DAPA	/1a(X)/, /13/	5.65	0.7	0.0085	0.62	0.99940

*eq. 4: $k_{1c} = k_{1D}$ (mm d^{-1}); $k_{2c} = k_{2D}$ (d^{-1}); eq. 14: Q = Q_{D2} (d^{-1}); eq. 15: Q = Q_{D3} (mm^{-1} d^{-1});
eq. 1a(X), ref. colony biomass: $k_{1c} = k_{1X}$ (μg$^{1/3}$ d^{-1}); $k_{2c} = k_{2X}$ (d^{-1}); $k_{3c} = k_{3X}$ (d^{-2}); Q = Q_{PX} (mm^2 μg^{-1} d^{-1})

Table 3 – *Comparison of different strains with respect to their kinetics parameters at different culture conditions: a) shaken flask cultures; b) laboratory bioreactor cultures; applied γ_{sfm} = 0.085 g L^{-1} (R6–500 and MV9R-2 strains)*

	k_1/(g$^{1/3}$ L$^{-1/3}$ h^{-1})	k_2/h^{-1}	K_x/g L^{-1}	k_4/ (g^{-1} h^{-2})	k_5/h^{-1}	K_s/g L^{-1}	q_{sm}/h^{-1}	q_{pm}/ h^{-1}	f_{ip}/L g^{-1}	q_{sf}/ h^{-1}	K_f/ g L^{-1}
a) R6–500	0.386	0.155	0.781			33	0.80	0.14	5.6	0.0009	0.006
MV9R-1	0.247	0.155	0.310	4.0	3.3	48	1.80	0.05	5.6		
MV9R-2	0.376	0.155	0.879			33	0.47	0.06	5.6	0.0005	0.006
b) R6–500	0.378	0.155	0.73			33	0.80	0.14	5.6	0.0009	0.006
MV9R-1	0.234	0.155	0.23	4.0	3.2	48	1.60	0.044	5.6		
MV9R-2	0.360	0.155	0.62			33	0.45	0.044	5.6	0.0005	0.006

Table 4 – *Correlation of computer simulation with experimental data. Determination coefficient (R^2) values*

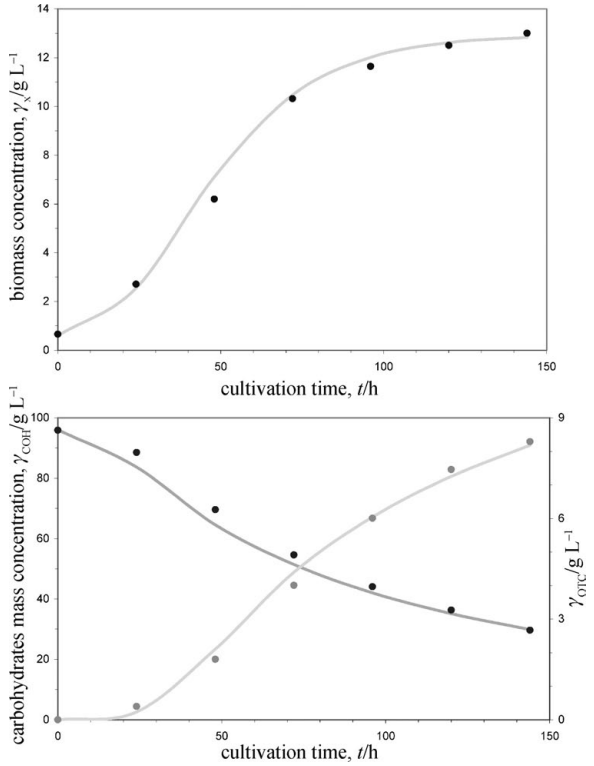
Microbial strain	Reaction system	Determination coefficient (R^2) for concen. of:		
		biomass	substrate	product
S.rimosus R6–500	shaken flask cultures	0.9943	0.9934	0.9956
S.rimosus R6–500	laboratory bioreactor culture	0.9962	0.9944	0.9981
S.rimosus MV9R-1	shaken flask cultures	0.9986	0.9973	0.9962
S.rimosus MV9R-1	laboratory bioreactor culture	0.9946	0.9921	0.9850
S.rimosus MV9R-2	shaken flask cultures	0.9969	0.9994	0.9988
S.rimosus MV9R-2	laboratory bioreactor culture	0.9969	0.9960	0.9977

Table 4a – *Correlation of computer simulation with experimental data. Coefficients of linear regression for the equation:* $X_{ex} = a\ X_{sim} + b$.

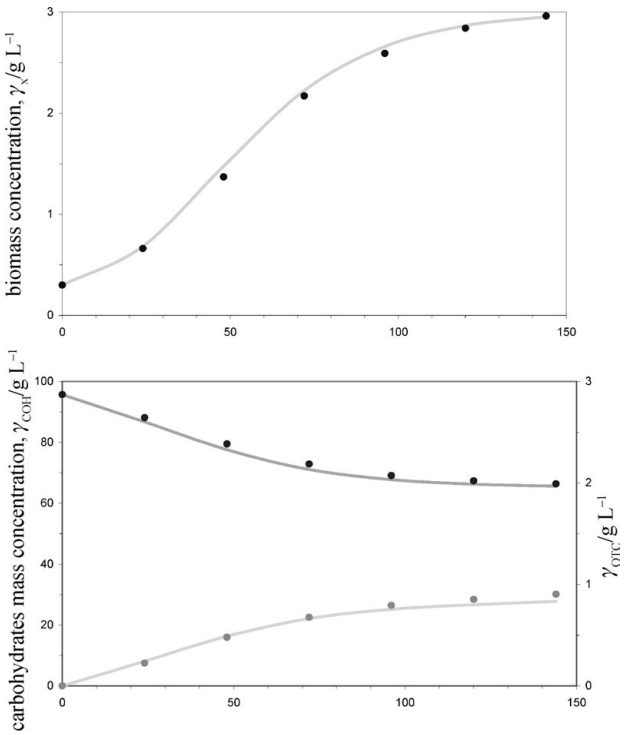
Microbial strain	Reaction system	Coefficients of linear regression for:		
		biomass	surrbstrate	product
S.rimosus R6–500	shaken flask cultures	$a =$ 1.0057	1.0230	1.0310
		$b =$ 0.1192	–0.8950	0.9490
S.rimosus R6–500	laboratory bioreactor culture	$a =$ 0.9972	0.9720	0.9743
		$b =$ –0.0340	1.1417	–0.0089
S.rimosus MV9R-1	shaken flask cultures	$a =$ 1.0013	1.0215	0.9195
		$b =$ 0.0363	2.8341	0.0247
S.rimosus MV9R-1	laboratory bioreactor culture	$a =$ 0.9780	0.9998	0.9729
		$b =$ 0.0628	0.1769	0.0246
S.rimosus MV9R-2	shaken flask cultures	$a =$ 0.9942	1.0410	1.0333
		$b =$ 0.0738	–3.5989	–0.0985
S.rimosus MV9R-2	laboratory bioreactor culture	$a =$ 0.9982	0.9559	0.9489
		$b =$ –0.0073	2.7648	0.0636

In Table 3 the values of kinetic parameters applied in computer simulations are summarised, indicating the kinetic characteristics of particular strains. However, the same k_2 values represent a common characteristics for all three strains, regardless of whether shaken flask cultures or cultures in laboratory bioreactors are considered. In contrast, parameter value differences caused by slight differences in applied cultivation conditions can be neglected to high extent. Since correlation coefficients (R) and determination coefficients (R^2) calculated to express a quality of the agreement between theoretical and experimental data were found to be excellent (R^2 values: for biomass concentrations, 0.9946 to 0.9986; for substrate concentrations, 0.9921 to 0.9994; for product concentrations, 0.9850 to 0.9988) in all presented cases (Table 4), and since the line coefficients resulting from linear regression analysis appeared do be quite acceptable (Table 4a), one can conclude that the applied mathematical models can be accepted and therefore recommended for the use in further studies.

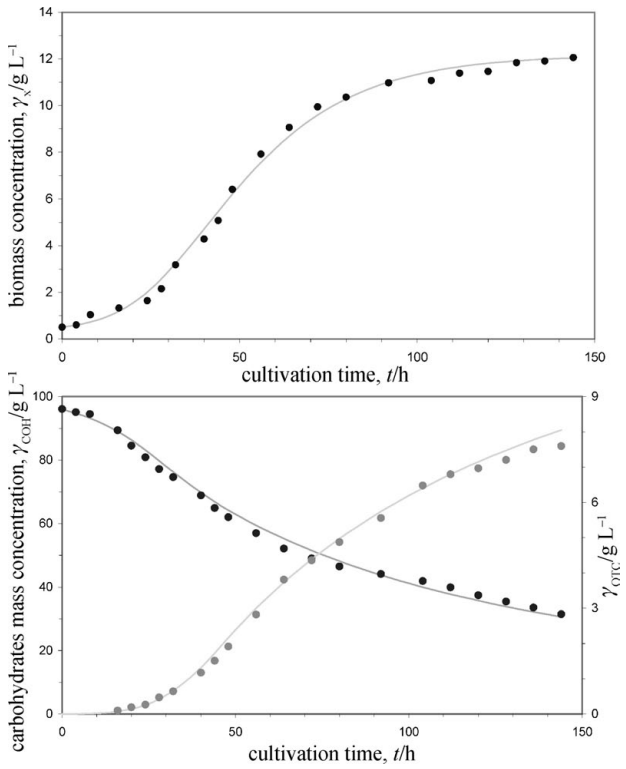
Disregarding the good agreement of theoretical with experimental data it could be useful to put some remarks. As known and previously mentioned,[29] different approaches to mathematical modelling can be applied and any mathematical model represents an oversimplified description of realty.



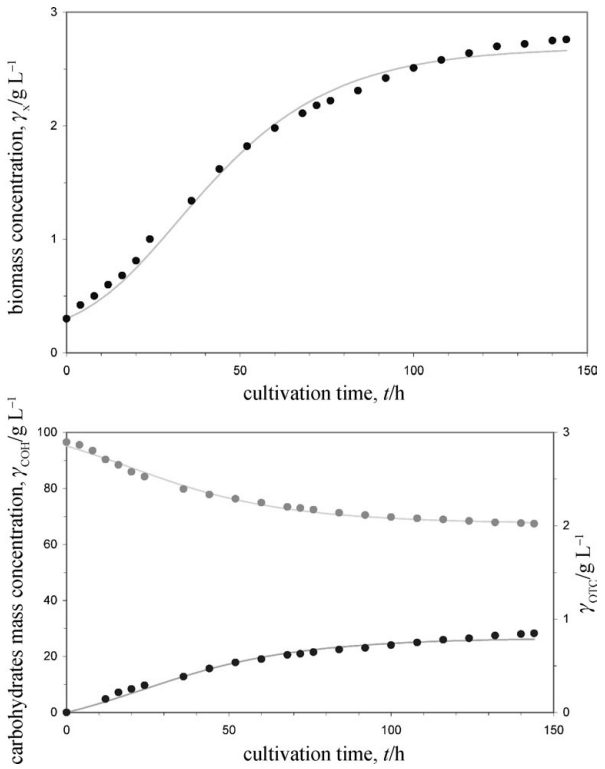
F i g . 1 – *Process kinetics in shaken flask cultures of Streptomyces rimosus R6–500. Points: experimental data; curves: computer simulation data; x-axis: cultivation time; y1-axis: biomass concentration; y2-axis: carbohydrate concentration; y3-axis: oxytetracycline concentration*



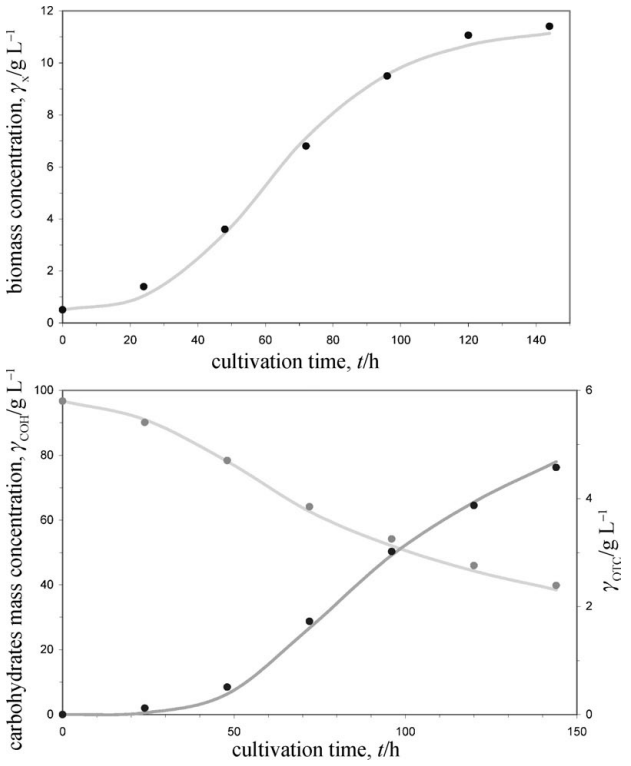
F i g . 3 – *Process kinetics in shaken flask cultures of Streptomyces rimosus MV9R-1. Points: experimental data; curves: computer simulation data; x-axis: cultivation time; y1-axis: biomass concentration; y2-axis: carbohydrate mass concentration; y3-axis: oxytetracycline mass concentration*



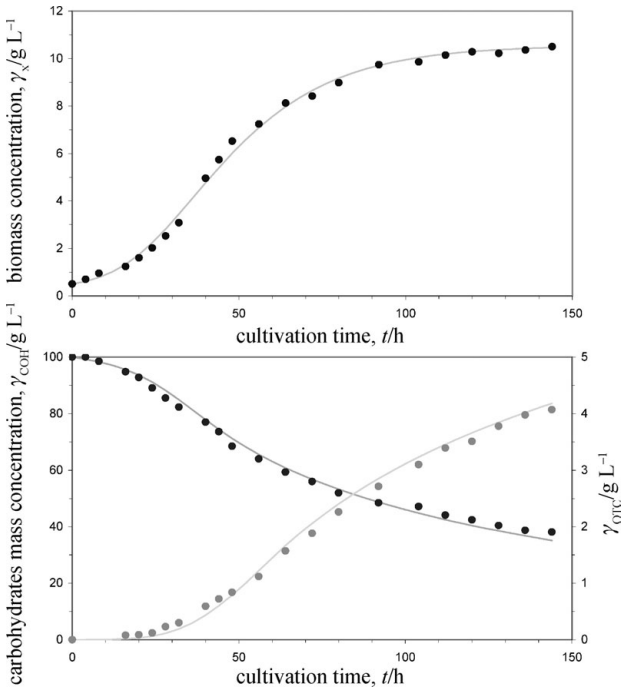
F i g . 2 – *Process kinetics in laboratory bioreactor culture of Streptomyces rimosus R6–500. Points: experimental data; curves: computer simulation data; x-axis: cultivation time; y1-axis: biomass concentration; y2-axis: carbohydrate concentration; y3-axis: oxytetracycline concentration*



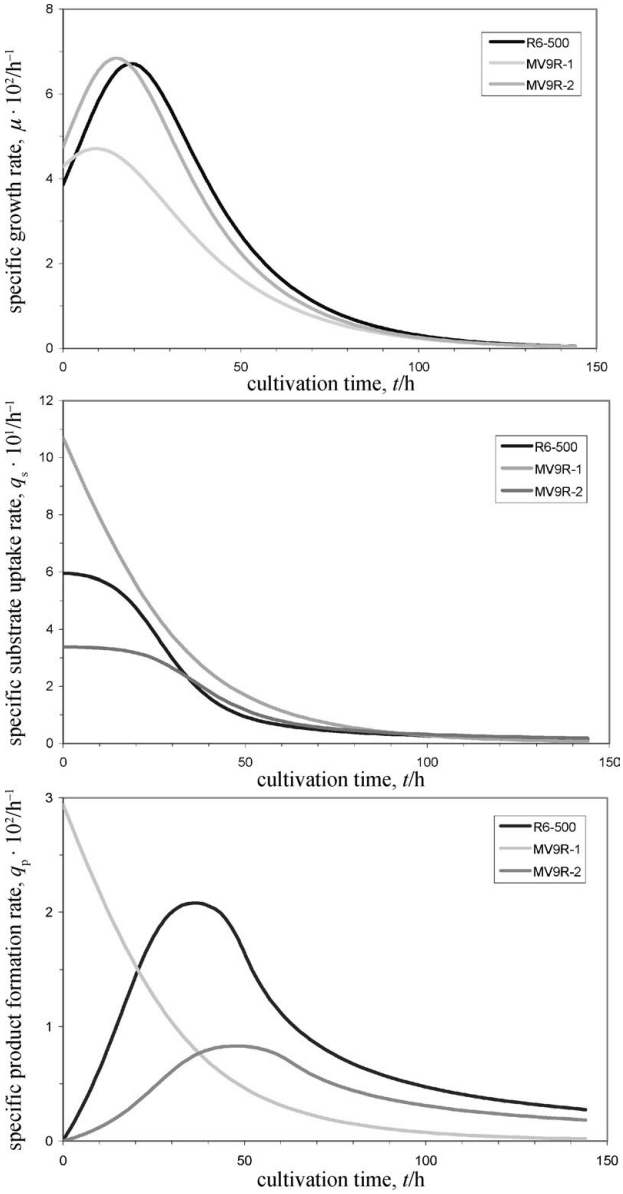
F i g . 4 – *Process kinetics in laboratory bioreactor culture of Streptomyces rimosus MV9R-1. Points: experimental data; curves: computer simulation data; x-axis: cultivation time; y1-axis: biomass concentration; y2-axis: carbohydrate mass concentration; y3-axis: oxytetracycline mass concentration*



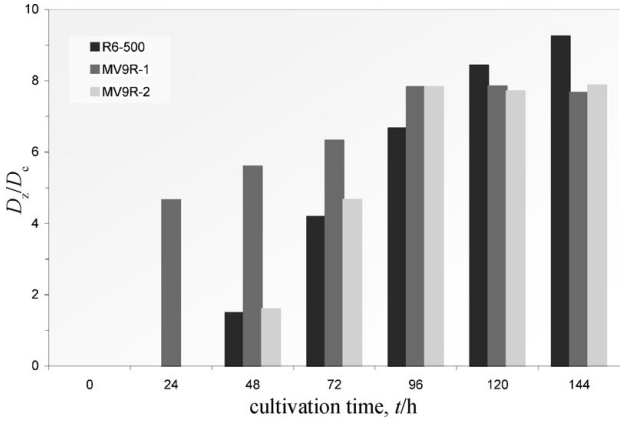
F i g . 5 – *Process kinetics in shaken flask cultures of Streptomyces rimosus MV9R-2. Points: experimental data; curves: computer simulation data; x-axis: cultivation time; y1-axis: biomass mass concentration; y2-axis: carbohydrate mass concentration; y3-axis: oxytetracycline mass concentration*



F i g . 6 – *Process kinetics in laboratory bioreactor culture of Streptomyces rimosus MV9R-2. Points: experimental data; curves: computer simulation data; x-axis: cultivation time; y1-axis: biomass concentration; y2-axis: carbohydrate mass concentration; y3-axis: oxytetracycline mass concentration*



F i g . 7 – *Theoretical (simulated) specific growth rates (a), specific substrate uptake rates (b) and specific product formation rates (c) in cultures of different Streptomyces rimosus strains*



F i g . 8 – *Changes of potency index (Ip = Dz/Dc) values of particular Streptomyces rimosus strains as the function of colony age*

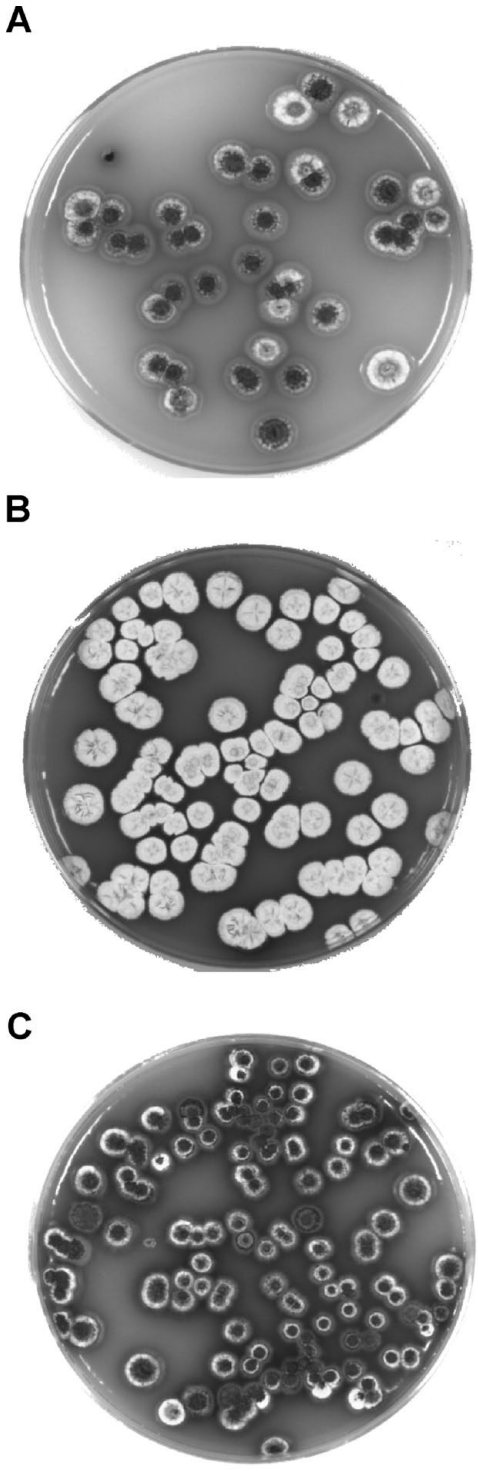


Fig. 9 – *Morphology of colonies after 10 d cultivation at 28 °C : A) S. rimosus R6–500;* ***B)*** *S.rimosus MV9R-1;* ***C)*** *S. rimosus MV9R-2*

This is especially valid when systems with live organisms are studied. Therefore, in the performed experiments one cannot absolutely exclude possibly overlooked deviations from perfect experiment realization. However, one can suppose the consequences of eventual such deviations reflected similarly to all investigated strains.

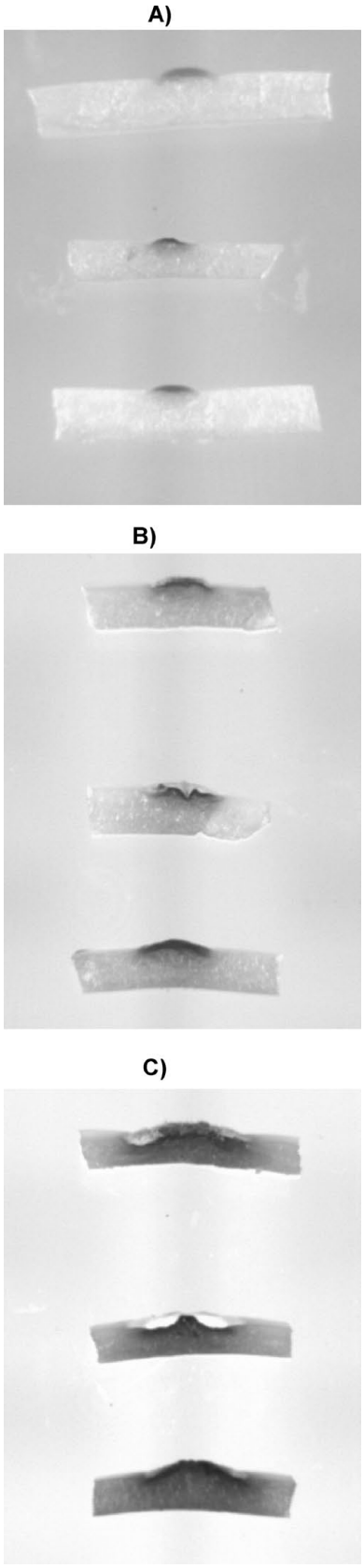


Fig. 10 – *Axial cross-sections of the colonies of S.rimosus R6–500, S.rimosus MV9R-1 and S.rimosus MV9R-2 (in descending order):* ***A)*** *4 d upon,* ***B)*** *6 d upon and* ***C)*** *10 d upon inoculation on agar-medium SM*

Finally, perhaps one can explain why the strain *S. rimosus* MV9R-1 appeared to be inferior to other two strains, presenting a more specifically behaviour. If potency index would be applied as criterion in screening the most productive microbial colonies, the strain MV9R-1 could be selected as superior to other two, if specific activities of younger colonies would be compared. If colonies of higher age would be compared, then the strain *S. rimosus* R6–500 would be selected as a superior one (Fig. 8). However, if morphology of particular strain colonies would be compared (Fig. 9, Fig. 10) then it becomes evident that colonies of MV9R-1 appear to be much more sporulated than colonies of other two strains. Since supposedly the spores are much less (if at all) active with respect to substrate uptake and antibiotic synthesis than vegetative cells, the more pronounced sporulation ability could be considered to be at least one of the causes determining a lower productivity of the strain MV9R-1 in its submerse cultures. *Hranueli* et al.[30] studied the processes of alkaline proteinase and α-amylase productions by corresponding *Bacillus* sp. strains and found that asporogenic mutants were more efficient than the starting sporogenic strains they derived from. Perhaps, such a result can support the previous explanation of specific behaviour of the strain *S. rimosus* MV9R-1. Therefore, it follows that potency index cannot be considered as a reliable criterion in screening antibiotic producing strains. Data in Fig. 8 suggest that a better criterion probably might be the rate of potency index increase during appropriate cultivation period. Such a criterion would be by its meaning closer to that proposed by *Trilli* et al.,[31] *i.e.* to specific product formation rate (Q). As already has been demonstrated,[9] the rate of potency index increase was higher for the strain 1001, expressing higher specific product formation rate Q, than for the strain 1003 expressing lower specific product formation rate (Tab. 2). Theoretically (eqs. 12–15), the specific product formation rate (Q) really appears to be the most appropriate criterion in evaluating microbial strain efficiency with respect to antibiotic production. However, microorganism morphology should be taken into consideration as well. Different morphological parameters of mentioned three strains were investigated.[32] In Fig. 10 the axial cross-sections of microbial colonies of different age are presented. The obtained results suggest that size, form and other characteristics of presented cross-sections of colonies corroborate to the conclusion that strain behaviours in their submerse cultures appear to be also a consequence of morphological strain characteristics. Since differences between cultures of different strains can be well defined by parameter values of applied kinetic models, one can conclude that differences in morphology are at least partly reflected in the kinetic parameter values. Specific properties of the strain MV9R-1 were expressed also through the value of parameter K_s, which refers to carbohydrates as substrate. In real reaction systems one can consider, for given starting nutrient medium, that value of the constant K_s can be affected by other components present in the medium. In the case of the strain MV9R-1 the higher K_s value could be a consequence of lack of some substance necessary for a normal metabolic activity of this strain cells.

Morphological characteristics can be expressed differently, by means of a series of indices. *S. rimosus* strains, *e.g.,* can differ with respect to the fractions of aerial and substrate mycelia, as was observed when strains 1001 and 1003 were compared (M. Bošnjak and J. Pigac, unpublished findings). Substrate mycelium was much more expressed at the strain 1003, which showed to be inferior when cultivated as microbial colony, especially when thick agarised media layers were applied. Availability of inorganic phosphate and dissolved oxygen disappearance around substrate mycelium can be considered to be the cause for reduced biosynthetic activity of colonies of the 1003 strain. To verify whether such an explanation could be accepted the application of agarised media layers fortified with inert metallic nets (screens) can be recommended in performing the experiments with microbial colonies.

For the process of industrial production of oxytetracycline it was demonstrated[33] that the actions oriented towards the optimal production process monitoring and especially to the optimal process control during every production process phase certainly can be fruitful and therefore recommendable. Results of present study can be applied to optimise the industrial production of oxytetracycline, and to support the process monitoring and the optimal control policies. However, the efforts oriented towards the efficient screening of microorganisms can also lead to improving the antibiotic production. Consequently, an adequate kinetic model describing the behaviour of antibiotic producing strains, derived under various culture conditions, can be a worthy instrument for an efficient process optimization.

Conclusion

Kinetics distinction among microbial strains of *Streptomyces rimosus* can be expressed by deterministic mathematical models developed on the basis of three-dimensional growth concept. Good agreement of theoretical with experimental data suggests that one can expect for microorganisms in general that adequately developed specific mathematical models can be applied in describing kinetics distinction among microbial strains.

List of symbols

D – diameter, L

D_0 – initial particle (colony) diameter, L

D_m – maximal particle (colony) diameter, L

D_c – microbial colony diameter, L

D_z – diameter of inhibition zone (surface area where test organism does not grow), L

D_{za} – diameter of inhibition zone formed due to antibiotic presence, L

f_{ip} – proportionality factor referring to inhibitory effect of formed product; inhibition constant, L^3M^{-1}

f_{kla} – propotionality factor referring to the effect of biomass on the reduction of oxygen dissolution rate, L^3M^{-1}

i – integer

I_p – potency index, dimensionless

K_f – saturation constant referring to inorganic phosphorus concentration, ML^{-3}

K_{OL} – saturation constant referring to dissolved oxygen concentration, ML^{-3}

K_s – saturation constant referring to substrate (carbohydrate) concentration, ML^{-3}

K_x – biomass adaptation constant; critical biomass concentration referring to growth initiation, ML^{-3}

k_1 – cubic growth rate coefficient, $M^{1/3}\ L^{-1}\ T^{-1}$

k_2 – coefficient of growth rate suppression, T^{-1}

k_3 – coefficient of growth rate retardation, T^{-2}

k_4 – coefficient of inactive biomass formation rate, $M^{-1}\ T^{-2}$

k_5 – coefficient of inactive biomass reduction rate, T^{-1}

k_{1D} – coefficient; maximal rate of colony diameter increase, $L\ T^{-1}$

k_{2D} – coefficient of suppression of colony linear growth, T^{-1}

k_{1X} – cubic growth rate coefficient of colony, $M^{1/3}\ T^{-1}$

k_{2X} – coefficient of colony growth rate suppression, T^{-1}

k_{3X} – coefficient of colony growth rate retardation, T^{-2}

k_La – volumetric coefficient of dissolved oxygen transfer rate, T^{-1}

n – integer

P – product mass; mass of antibiotic produced by colony, M

p_i – mean antibiotic concentration in inhibition zone ring between R_i and R_{i-1}, $M\ L^{-2}$

Q – specific rate of product formation in general meaning; specific meaning and dimensions one defines by adding indices

Q_{D2} – rate constant of product zone linear increase, T^{-1}

Q_{D3} – rate constant of product zone linear increase, $L^{-1}\ T^{-1}$

Q_p – specific rate of product formation; constant of non-growth associated product formation rate, T^{-1}

Q_{px} – specific rate of product zone (inhibition zone) linear increase; rate constant of product zone linear increase, $L^2\ M^{-1}\ T^{-1}$

q_{O2} – specific oxygen uptake rate; respiration coefficient, T^{-1}

q_p – specific product formation rate, T^{-1}

q_{pm} – maximal specific product formation rate, T^{-1}

q_s – specific substrate uptake rate, T^{-1}

q_{sf} – specific uptake rate of inorganic phosphorus, T^{-1}

q_{sm} – maximal specific substrate uptake rate, T^{-1}

R_i – distance from the colony centre, L

S_i – circle surface area, L^2

t – cultivation time, T

X – biomass (colony biomass), M

Greek letters

α_i – proportionality factor referring to inhibitory effect of formed product, dimensionless

γ_{OL} – dissolved oxygen concentration, ML^{-3}

γ_{OL}^* – oxygen solubility or saturation dissolved oxygen concentration, ML^{-3}

γ_p – product (antibiotic) mass concentration, ML^{-3}

γ_s – substrate mass concentration, ML^{-3}

γ_{sf} – inorganic phosphorus mass concentration, ML^{-3}

γ_{sfm} – maximal inorganic phosphorus mass concentration; constant, ML^{-3}

γ_x – microbial biomass concentration, ML^{-3}

γ_{xac} – active biomass concentration, ML^{-3}

γ_{xin} – apparently inactive biomass concentration, ML^{-3}

Φ_s – physiological factor referring to substrate uptake, dimensionless

Φ_x – physiological factor of biomass adaptation to reproduction activity, dimensionless

Abbreviations

C.F.U. – colony forming unit;

DAPA – α,ε– L,L – diaminopimelic acid;

MA – malt agar medium;

MAYE – malt agar -yeast extract medium;

MC – maltose – CSL medium;

MCYE – maltose – CSL – yeast extract medium;

MIC – medium for inocula preparation;

MOP – medium for oxytetracycline production;

OTC – oxytetracycline;

SM – soya – manitol -agar medium

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