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EXPERIMENTAL ARTICLES

Increase of 1-Hydroxy-2-Naphthoic Acid Concentration as a Cause of Temporary Cessation of Growth for *Arthrobacter* sp. K3: Kinetic Analysis

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Abstract—Consumption of 1-hydroxy-2-naphthoic acid by strain *Arthrobacter* sp. K3 was investigated. Drastic increase in the substrate concentration in flow culture was shown to induce the lag phase of growth in case the initial substrate concentration in the medium was not saturating; the culture originally saturated with the substrate ($S \gg K_S$) was resistant to the concentration increase. In accordance with the constructed kinetic model, lag phase results from an accumulation of intermediates in the metabolic system.

Key words: lag phase of growth, adaptation to toxic substrates, kinetic analysis, 1-hydroxy-2-naphthoic acid, substrate constant.

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From the kinetic point of view, microbiological processes may be either stationary or nonstationary [1]. In a stationary process, all the culture parameters (enzymatic activities, concentrations of metabolites, metabolic fluxes, and specific growth rate) are completely determined by current values of the medium parameters (substrate concentrations, temperature, etc.). In a nonstationary process, the medium parameters change so rapidly that the state of the culture depends not only on the current medium parameters but also on the prehistory of the system. The term "lag phase" is used to define a nonstationary process in a description of microbial growth. Lag phase in the broad sense is a delayed response of a microbial population to abrupt environmental changes [2]: growth inhibition after inoculation into fresh medium; temporary growth cessation in cases of diauxy, abrupt change in temperature or medium composition; delayed inactivation of the culture. Understanding of lag phase mechanisms is of great practical significance for preservation of foodstuffs, efficient bioremediation, and maintaining stability in the processes of wastewater purification and microbiological synthesis.

Lag phase duration depends on the preceding cultivation conditions: as a rule, the higher the rate of change for environmental parameters, the longer the lag phase [3, 4]. The causes of the lag phase are defined as the need for induction of an appropriate enzymatic system, transformation of a pre-substrate into a substrate, and removal of growth inhibitors [5]. A rather general approach to the analysis of nonstationary microbiological processes was proposed by Powell [1]. In some publications, the lag phase model was based on the idea of induction of an enzymatic system as a first-order reaction [6] or an autocatalytic process [7]. Stochastic lag phase models [8] were constructed in consideration of cell population heterogeneity.

The goal of this work was to study the lag phase of growth of *Arthrobacter* sp. K3 culture observed after an abrupt increase in concentration of the growth substrate: 1-hydroxy-2-naphthoic acid (1H2NA).

MATERIALS AND METHODS

Strain. Strain *Arthrobacter* sp. K3 from the collection of The Laboratory of Enzymatic Degradation of Organic Compounds, Institute of Biochemistry and Physiology of Microorganisms Russian Academy of Sciences, was used in the work. The strain can utilize phenanthrene as the sole carbon and energy source producing 1H2NA as an intermediate; it is an *L*-methionine auxotroph; the strain was characterized in more detail in the work [9]. Methanol and methionine, included in the medium in our experiments, are not utilized by the culture as carbon and energy sources (the biomass yield is proportional to the initial 1H2NA concentration).

Incubation medium. The mineral medium contained the following (g/l): NH_4NO_3, 1.0; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; $FeCl_3$, 2 drops of the saturated solution, pH ~7.5. The mineral medium was filtered and sterilized. After sterilization,

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L-methionine (20 mg/l) was added as a sterile aqueous solution in the concentration of 2 g/l.

The required 1H2NA concentration was obtained by introduction of 1H2NA solution in methanol (1 ml of 1H2NA solution per liter of the medium).

Measurement of 1H2NA concentration. For the measurement of 1H2NA concentration, a culture liquid sample (4 ml) was collected; after centrifugation, the clear supernatant was transferred into a cuvette, and the UV spectrum was taken in a Shimadzu UV160 spectrophotometer. Absorption at 250 nm was proportional to 1H2NA concentration in the incubation medium; the molar extinction was $\varepsilon_{250} = 3.4 \times 10^5$ (M cm)$^{-1}$. The analysis of UV spectrum dynamics at 1H2NA conversion by the culture of *Arthrobacter* sp. K3 showed that no intermediates absorbing in the UV range were accumulated in the medium during this process (the spectra were invariant relative to compression along the axis of optical density). Consequently, 1H2NA concentration in the culture liquid may be calculated from the spectrophotometry data using the formula $S = \frac{D_{250}}{\varepsilon_{250}} = \frac{D_{250}}{3.4 \times 10^5}$ (mol/l).

Growth curve plotting. For assessment of biomass concentration by turbidity, optical density of the culture liquid was measured in a KFK colorimeter with a green light filter (540 nm) in a 1-cm thick cuvette.

Continuous cultivation was performed at 29°C in a fermentor with the working volume of 0.45 l; the mineral medium containing 1H2NA (266 μM = 50 mg/l) and L-methionine (20 mg/l) was fed by a peristaltic pump at a predetermined rate. The culture was intensively stirred by a magnetic stirrer and drained from the fermentor at a rate equal to the medium's supply rate. The measurement of washout dynamics [10] showed that the system satisfied the model for a flow reactor with ideal stirring.

Experiments on 1H2NA addition to the culture liquid from the flow culture. After the steady-state 1H2NA concentration was established in the flow culture, the medium feed was switched off and the culture from the fermentor was distributed into four flasks (100 ml per each) followed by an addition of 100 μl of 1H2NA solution in methanol in the concentrations of 33, 66, 133, and 266 mM. The flasks were placed on a shaker and cultivated at 29°C with periodical sampling to measure the culture turbidity and 1H2NA concentration.

Substrate constant estimation. The data on 1H2NA consumption at low substrate concentrations were smoothed by the Henri's curve $t = \frac{K_S}{v_m}\ln\left(\frac{S_0}{S}\right) + \frac{1}{v_m}(S_0 - S)$, which is an integral of the Michaelis–Menten equation; the parameters v_m and K_S were determined in the Foster–Niemann coordinates $\left(\frac{\ln S_0 - \ln S}{t}; \frac{S_0 - S}{t}\right)$ [11].

RESULTS

1H2NA consumption in a batch culture. The strain *Arthrobacter* sp. K3 can utilize 1H2NA as a sole carbon and energy source. The batch culture grew exponentially ($\mu_m = 0.08 \pm 0.02$ h^{-1}, $n = 5$, $p = 0.95$). The typical growth curve is shown in Fig. 1. The decrease of 1H2NA concentration from 800 to 400 μM (Fig. 1a) was accompanied by culture growth at a constant specific rate $\mu_m = 0.07$ h^{-1} (Fig. 1b) and constant biomass yield $Y_S = 0.0002$ (optical U/μM) (Fig. 1c). Thus, 1H2NA consumption by strain *Arthrobacter* sp. K3 did not depend on the substrate concentration (in the relevant range of substrate concentrations) and was not inhibited by metabolic products.

At low substrate concentrations (about 10 μM), the curve of 1H2NA consumption by *Arthrobacter* sp. K3 is a typical kinetic curve for a reaction obeying the Michaelis kinetics (Fig. 2); the substrate constant is $K_S = 5.4$ μM.

1H2NA consumption in a continuous culture. A few days after activation of the flow in continuous culture of *Arthrobacter* sp. K3, the steady-state value of 1H2NA concentration was established; however, the system did not satisfy the classical chemostat model (probably, due to formation of a biofilm on fermentor walls). If the medium feed rate was enhanced after the steady-state condition was achieved, 1H2NA concentration in the fermentor increased sharply and then decreased to a new steady-state level (Fig. 3); the observed effect apparently resulted from temporary inhibition of growth and 1H2NA consumption (lag phase).

Lag phase at 1H2NA addition to the culture liquid. For elucidation of the causes for lag phase observed at an increase of the medium feed rate, different amounts of 1H2NA were added to aliquots taken from the steady-state flow culture and the dynamics of turbidity was followed during the incubation of the resultant batch cultures. Growth depended on the steady-state concentration of 1H2NA in the fermentor ($\tilde{S}$), from which the culture was taken (Fig. 4). The initial parts of the growth curves matched each other $\tilde{S} = 13$ μM (Fig. 4c), while growth inhibition depending on the amount of added 1H2NA was observed at $\tilde{S} = 5$–6 μM (Fig. 4a, b); at the same time, the analysis showed that the lag phase (λ) was directly proportional to the change in 1H2NA concentration (Fig. 5).

At an increase in the steady-state substrate concentration in the continuous culture from which the aliquot was taken, from 3.77 to 13.00 μM, the coefficient of

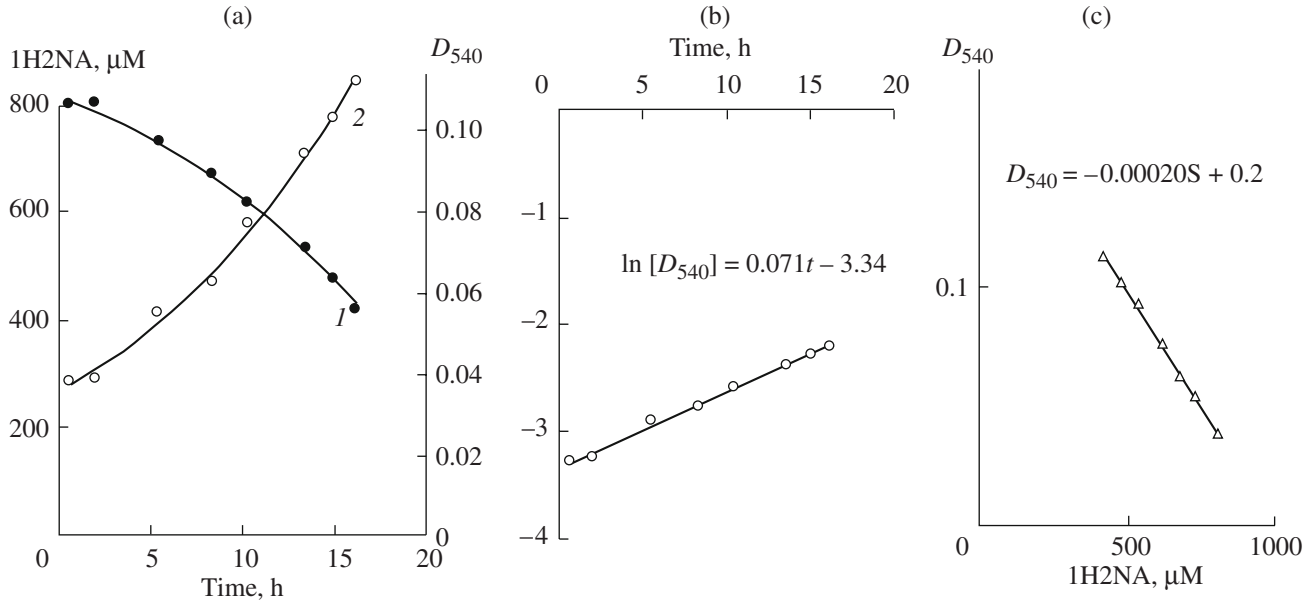

Fig. 1. Growth of the culture of *Arthrobacter* sp. K3 in the mineral medium with 1-hydroxy-2-naphthoic acid and L-methionine (20 mg/l). a, Consumption of 1H2NA (*1*) and culture growth (*2*); b, Growth curve in semi-logarithmic coordinates; c, Dependence of biomass concentration on 1H2NA concentration.

proportionality between the jump in 1H2NA concentration and the lag phase $\frac{\lambda}{\Delta S}$ decreased from 2.65 (min/μM) to zero (Fig. 6). It implies that at a 1H2NA concentration in the medium above ~10^{-5} M, no increase in 1H2NA concentration would result in the lag phase.

Fig. 7 shows the relative specific rate of 1H2NA consumption depending on 1H2NA concentration in the culture liquid, calculated by equation $\frac{q}{q_m} = \frac{S}{K_S + S}$ using the experimentally determined substrate constant value. The same figure presents the dependence of the coefficient of proportionality between the 1H2NA concentration increase and lag phase duration on 1H2NA concentration. Comparison of the plots shows a severe decrease of $\frac{\lambda}{\Delta S}$ at 1H2NA concentrations close to the substrate constant. The culture growing with the maximal specific growth rate (i.e., saturated with the substrate) is absolutely resistant to drastic 1H2NA changes in the medium. Thus, an abrupt increase in substrate concentration in the flow culture of *Arthrobacter* sp. K3 not saturated with the substrate induces transition of the culture to the lag phase with duration directly proportional to the value of substrate concentration increase, whereas the culture saturated with the substrate is resistant to abrupt increases in substrate concentration.

Fig. 8 shows 1H2NA consumption by the culture of *Arthrobacter* sp. K3 in the state of lag phase. The rate of 1H2NA consumption during the lag phase was constant, by an order of magnitude less than in the growing culture, and did not depend on the amount of added 1H2NA that caused growth cessation. More pronounced increases in 1H2NA concentration corresponded to longer lag phases at equal rates of substrate consumption during the lag period.

DISCUSSION

1H2NA concentrations greatly exceeding the substrate constant ($S \gg K_S$) are kinetically indistinguishable, i.e. they lead to the same specific rate of substrate consumption; this is also true for the culture in the state of lag phase. However, lag phase duration depends on the increase in substrate concentration in the medium, $\Delta S = S_2 - S$ at $S_2 \gg K_S$, i.e. the increase in 1H2NA concentration from the same steady-state value S to different kinetically indistinguishable values $S_2^{(1)}$ and $S_2^{(2)}$, leads to different lag phase durations. Consequently, consideration for the steady-state kinetics of substrate consumption by the growing culture and the culture in the state of lag phase gives no answers concerning the cause of the lag phase; hence, it is necessary to consider the nonstationary process of culture transition from the growth to lag phase. We propose a simple kinetic model corresponding to the experimental data. Let us consider the system

$$S \underset{1}{\longleftrightarrow} S' \xrightarrow[2]{} M \xrightarrow[3]{} \ldots \qquad (1)$$

consisting of three consecutive reactions:

(1) Diffusion of the substrate to the place of conversion,

$$v_1 = k_1(S - S');$$

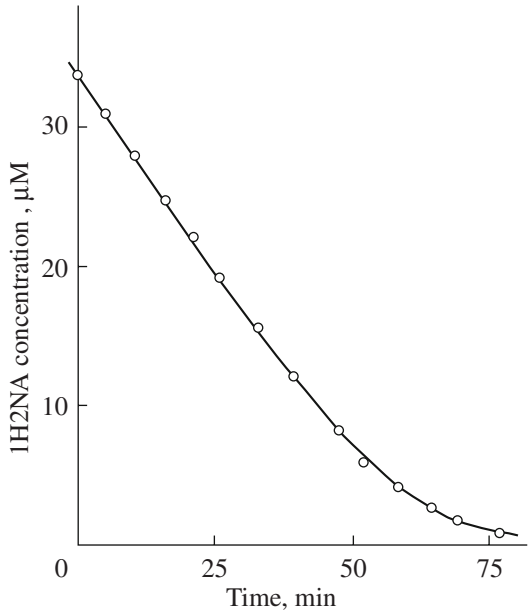

Fig. 2. 1H2NA consumption by the culture of *Arthrobacter* sp. K3 at low 1H2NA concentrations in the medium. The data are smoothed by the integral of equation $\frac{-dS}{dt} = \frac{v_m S}{K_S + S}$, as described in the section Substrate constant estimation.

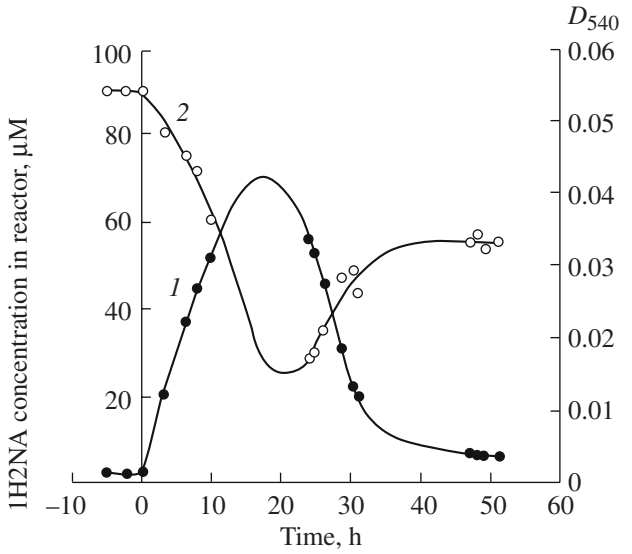

Fig. 3. Dynamics of 1H2NA concentration in the reactor (*1*) and culture turbidity (*2*) under continuous cultivation of *Arthrobacter* sp. K3 after changing the flow rate from 0.06 to 0.09 h^{-1}. The flow rate was changed at the moment of time $t = 0$ h.

(2) Conversion of the substrate into the intermediate *M*,

$$v_2 = \frac{v_{m2} S'}{K_2 + S'};$$

(3) Consumption of the intermediate *M*,

$$v_3 = \frac{v_{m3} M}{K_3 + M}.$$

Let us suppose that the steady flux through the system $\left(v = \frac{v_m S}{K_S + S}\right)$ is completely determined by the second stage ($v_{m2} = v_m$; $K_2 = K_S$). As is known from the kinetics of consecutive reactions, the first stage is close to equilibrium ($S = S'$) while the third stage functions in the first-order mode $\left(v_3 = \frac{v_{m3}}{K_3} M = k_3 M\right)$. Let system (1), being in the steady state, have a great positive increase in substrate concentration ($\Delta S \gg S$). Then, the first reaction begins to function in an irreversible mode and in time τ the substrate concentration in the site of its conversion increases by value

$$\delta S' = k_1 \Delta S \tau. \quad (2)$$

The intermediate *M* is a product of slow reaction and a substrate of a quick reaction, therefore quasi-steady approximation can be applied to its concentration:

$$v_2 = \frac{v_m S'}{K_S + S'} = k_3 M = v_3. \quad (3)$$

By differentiation of (3), we find an excess of the intermediate *M* formed during time τ:

$$\delta M = \frac{v_m K_S}{k_3 (K_S + S')^2} \delta S'. \quad (4)$$

If the second stage is completely blocked at time τ after the increase in the substrate concentration, then the consumption of excessive metabolite *M* requires a lag phase with the duration of

$$\lambda = \frac{\delta M}{k_3 M} = \frac{K_S}{k_3 (K_S + S')} \delta S' = \frac{K_S}{k_3 (K_S + S') S'} k \Delta S \tau. \quad (5)$$

Taking into account that $S' = S$, the ratio of the lag phase to the preceding substrate increase is determined by expression:

$$\frac{\lambda}{\Delta S} = \frac{b}{(K_S + S) S}, \quad (6)$$

where $b = \frac{k_1 K_S \tau}{k_3}$.

Function (6) is in the best agreement with the experimental data at parameter value $b = 54$ (dotted line in Fig. 7).

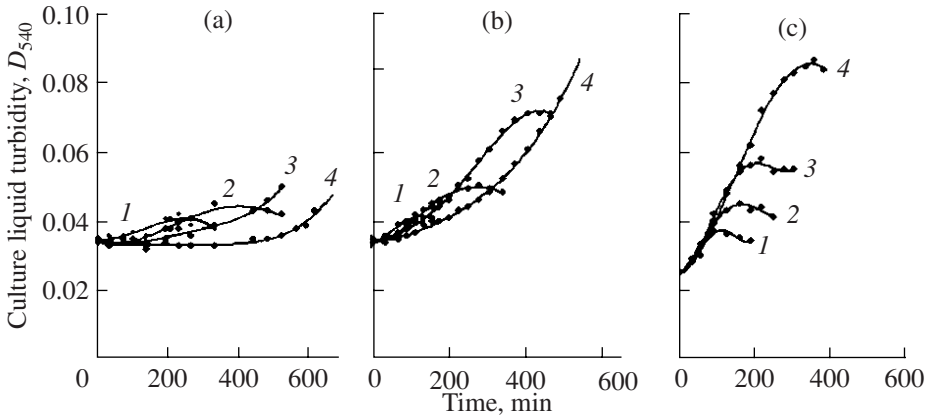

Fig. 4. Growth of batch cultures of *Arthrobacter* sp. K3 obtained by addition of 1H2NA to the aliquots taken from the flow culture. Steady-state substrate concentration in the flow culture was: 5.44 μM (a); 6.02 μM (b); and 13.00 μM (c). The substrate was added in the following concentrations: 33 μM (*1*); 66 μM (*2*); 133 μM (*3*); and 266 μM (*4*).

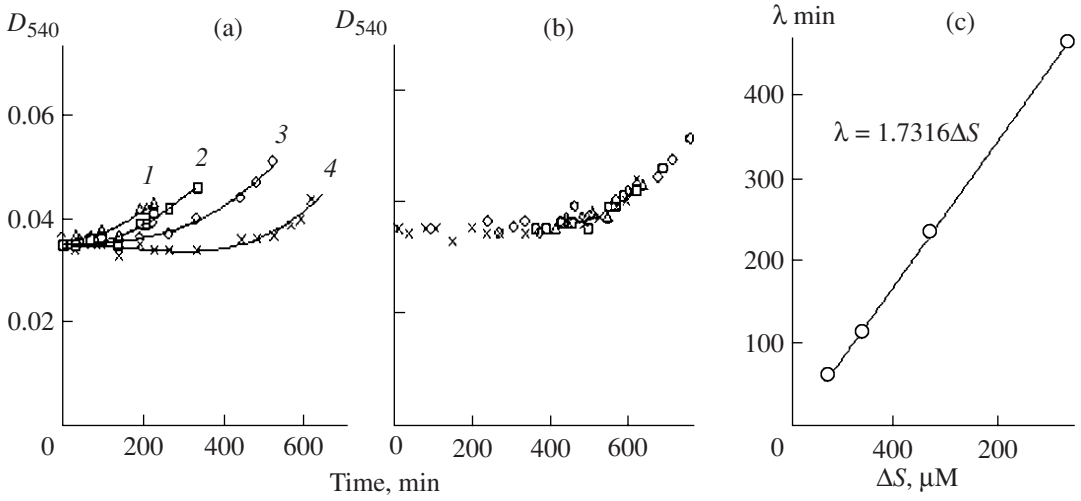

Fig. 5. Calculation of the lag phase of growth of *Arthrobacter* sp. K3 culture at different increases of 1H2NA concentration in the medium. a, Growth curves of *Arthrobacter* sp. K3 culture grown in the flow reactor at $\tilde{S}$ = 5.44 μM after addition of the substrate (μM): *1*, 33; *2*, 66; *3*, 133; *4*, 266. b, The same data aligned by parallel transfer along the time axis. c, Dependence of the lag phase of growth {λ} on substrate concentration increase {Δ*S*}. Lag phase was calculated as a shift of the given curve necessary for alignment of the curves by parallel transfer along the time axis, using the initial parts of growth curves (to the degree of substrate conversion no more than 50%). The linear dependence λ(Δ*S*) was converted into direct proportionality by normalization $\begin{cases} \Delta S = 0 \\ \lambda = 0 \end{cases}$.

We believe that after the increase in substrate concentration in the medium (Δ*S*) the events develop in the following way. Diffusion of the substrate leads to an increase in its concentration in the cells in proportion to Δ*S*. If the culture is not saturated with the substrate, the increase in the substrate concentration enhances the flux through the metabolic system and the concentration of the intermediates of 1H2NA metabolism in the latter, i.e. disturbs homeostasis. After a certain period of time τ, required for physiological response, feedback-type regulation suppresses substrate conversion and the culture passes to the lag phase. The excess of the intermediates (δ*M*) accumulated in the nonstationary mode (during the time τ) is proportional to the increase in the substrate concentration (Δ*S*) but depends also on the initial substrate concentration *S* (homeostasis is not disturbed in the culture saturated with the substrate). The lag phase serves as homeostasis recovery; the longer it is, the more excessive substance has been formed in the metabolic system.

Our experimental data and their analysis show that the culture saturated with the substrate ($S \gg K_S$) is resistant to the increases in its concentration. The essential difference between the cultures saturated and unsatur-

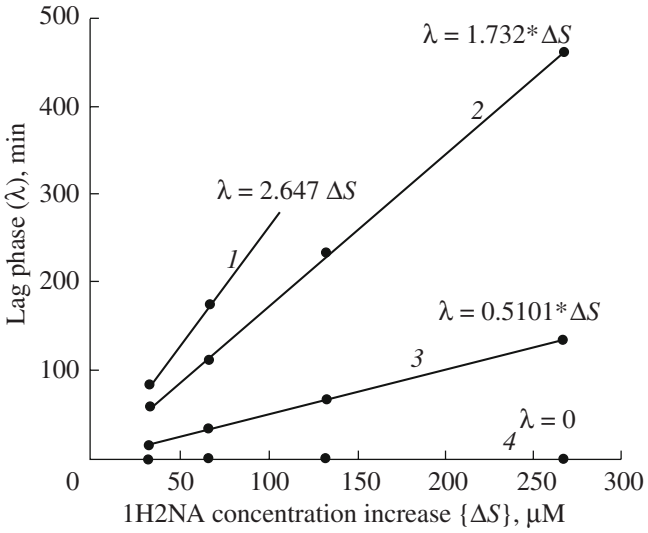

Fig. 6. Dependence of the lag phase of growth on the increase of 1H2NA concentration at the following steady-state 1H2NA concentrations in the flow culture (μM): 3.77 (*1*); 5.44 (*2*); 6.02 (*3*); and 13.00 (*4*).

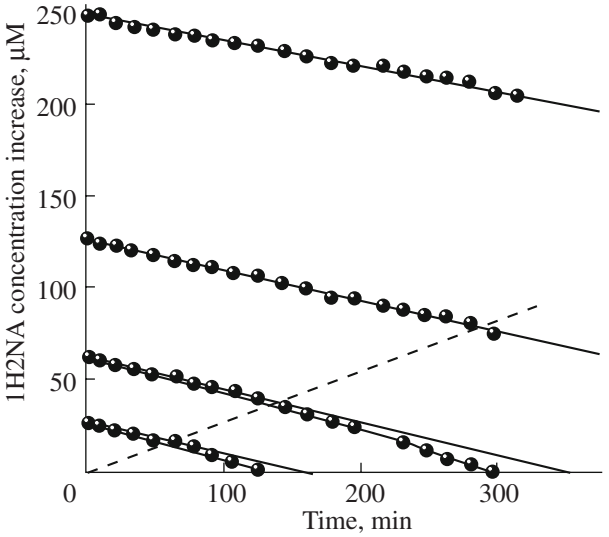

Fig. 8. The dynamics of 1H2NA concentration after 1H2NA addition to the aliquot taken from the flow culture at a steady-state 1H2NA concentration in the fermentor (3.77 μM). Intersection of the dotted line with the kinetic curve corresponds to the beginning of culture growth.

ated with the substrate is that no increase in the substrate concentration in the medium will ever result in the increase of the specific rate of substrate consumption by the substrate-saturated culture. The increase in substrate concentration in an unsaturated culture, on the contrary, inevitably leads to the increase of the specific rate of substrate consumption. If the metabolic system of substrate utilization is not ready for such a drastic increase of the flux, matter accumulates in the system in the form of certain intermediates; hence, substrate utilization is suppressed and the culture passes to the lag phase, which the longer, the more excessive substance is formed in the metabolic system. The significance of saturation of the metabolic system with the substrate for homeostasis maintenance was indicated in theoretical studies [12].

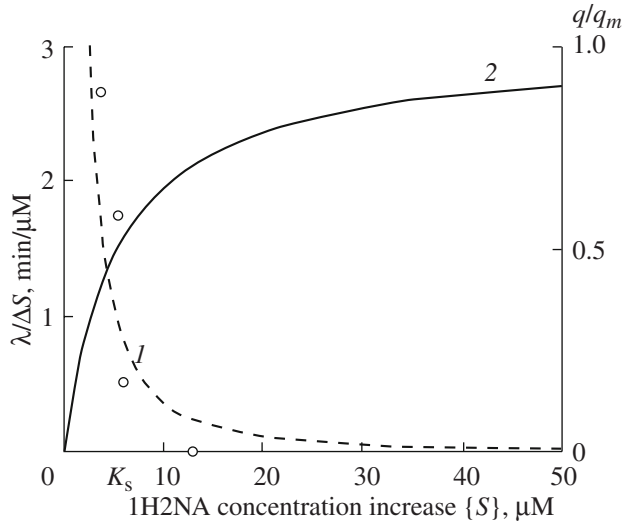

Fig. 7. Association of specific rate of 1H2NA consumption by strain *Arthrobacter* sp. K3 with resistance of the culture to abrupt changes of 1H2NA concentration in the medium. Dependence of the lag phase ratio to the preceding increase of 1H2NA concentration $\left(\frac{\lambda}{\Delta S}\right)$ on the steady-state 1H2NA concentration in the fermentor (*1*); dependence of the relative specific rate of 1H2NA consumption $\left(\frac{q}{q_m}\right)$ on 1H2NA concentration in the medium (calculated by the previously found value K_S) (*2*).

If the flow system similar to the one investigated in this work is used for wastewater purification, the incoming flux must provide the saturating substrate concentration in the reactor; otherwise, increased concentration in outflows will result in unstable work for the system (Fig. 3); therefore, the depth of purification in such a reactor is determined by the substrate constant of a relevant microorganism.

The question about the universal character of the examined lag phase mechanism needs further investigation. Probably, it underlies the behavior of "fickle" cultures that require long-term adaptation to a toxic substrate.

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