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MODELLING OF AEROBIC BIODEGRADATION OF PHENOL BY *Stenotrophomonas maltophilia* KB2 STRAIN

MODELOWANIE TLENOWEJ BIODEGRADACJI FENOLU PRZEZ SZCZEP *Stenotrophomonas maltophilia* KB2

Abstract: The microbial degradation of phenol by *Stenotrophomonas maltophilia* KB2 strain was studied. The purpose of experiments was to determine optimal environmental conditions for bacterial growth and to develop equation describing both the rate of cell growth and biodegradation rate of phenol. Microbial growth tests in the presence of phenol as the sole carbon and energy source were conducted in batch reactor for different initial concentration of the degraded compound changed in the range of 25-500 $g\cdot m^{-3}$. The kinetic experiments were performed in optimal environmental conditions (30ºC, pH 7). The Haldane inhibitory model with the values of constants: $\mu_m = 0.9$ h^{-1}, $K_s = 48.97$ $g\cdot m^{-3}$, $K_i = 256.12$ $g\cdot m^{-3}$ predicted the experimental data with the best accuracy. The obtained data-base made it possible also to determine the values of biomass yield coefficient, $(Y_{XS})_{obs} = 0.614$, and the endogenous decay coefficient, $k_d = 0.05$ h^{-1}.

Keywords: phenol, biodegradation, batch culture, kinetics

Introduction

Phenol and phenolic derivatives are one of the largest groups of environmental pollutants due to their presence in many industrial effluents and broad application as antibacterial and antifungal agents [1]. Phenolic compounds enter the environment through the decomposition of the attached algae, phytoplankton and through wastewater discharges from a variety of industries such as oil and petroleum refineries, coking plants, coal processing, industries of resins, paints, dyes, petrochemicals production, textiles and paper mills [2]. Phenols have been on the list of priority pollutants for a long time, due to causing many adverse effects to human health (toxic to the nervous system, the heart, the kidneys, the liver and are readily absorbed through skin and mucosa) and the environment. Concentrations as low as 0.005 $mg\cdot dm^{-3}$ cause adverse effect to aquatic environment, while 0.8 $mg\cdot kg^{-1}$ of soil is considered toxic [3].

Phenol is either toxic or lethal to fish at concentration of 5-20 $mg\cdot dm^{-3}$, and imparts medicinal taste and odour even at much lower concentration of 2 $\mu g\cdot dm^{-3}$. Therefore, to save our aqueous ecosystems, it has been mandatory for industries to treat these wastes before safe disposal to water bodies [4]. Various treatment methods are available for reduction of phenol content in wastewater. The physicochemical methods for the treatment of phenol laden wastewater include: chlorination, ozonation, adsorption, solvent extraction, membrane process, coagulation, flocculation [5]. The major disadvantages of the conventional treatments are costs involved in the disposal of the final effluent, production of toxic intermediates and incomplete mineralization of the compounds. In such cases, biological processes seem very promising in terms of complete mineralization of phenols to

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CO_2 and H_2O, without producing any toxic residues, low cost and efficiency of these methods [6]. In biodegradation of phenol the microorganisms play a key role. In spite of the toxicity of this compound, a number of microorganisms are able to use phenol as a source of carbon and energy. A lot of information is accumulated on bacterial species from the *Pseudomonas* genus [7-10] and *Bacillus* genus [2, 11, 12]. After many years of research into aromatic compound biodegradation, a number of other bacterial species have been also described such as *Ewingella Americana* [13], *Staphylococcus aureus* [14], *Alcaligenes faecalis* [15] or mixed bacteria cultures obtained from activated sludge [6, 16-18].

Despite the significant amount of information gathered during the years the problem is still topical and significant and stimulates research being aimed at characterization of new microbial species which successfully utilize phenol as a single carbon source. Therefore, the subject of research in the presented study was to determine kinetics of phenol biodegradation by a novel strain of bacteria, identified as *Stenotrophomonas maltophilia* and designated as KB2. The ability of this strain to use phenol as the sole carbon and energy source is connected with the presence of special enzyme - phenol monooxygenase, which takes part in the first step of phenol metabolic pathway [19-21].

The aim of the studies was to determine the optimal conditions for growth selected microorganisms and next to develop equations describing the rate of growth of microorganisms and the biodegradation of the utilized substrate.

Batch experiments

The kinetic model parameters are usually obtained from batch experiments by observing the biomass growth rate with time at different initial concentration of the substrate. For the exponential growth phase, where the specific growth rate of biomass (μ_{net}) remains constant and its value depends only on the initial substrate level, the rate of changes in biomass concentration is first order and can be defined as:

$$\frac{dX}{dt} = \mu_{net} X \tag{1}$$

where μ_{net} is net specific growth rate [h^{-1}] and X is biomass concentration [$g \cdot m^{-3}$].

Integrating equation (1) with known initial condition $X = X_0$ at $t = 0$ yields:

$$\ln X = \mu_{net} t + \ln X_0 \tag{2}$$

For every experimental point, that is for every initial dose of substrate, the graph representing the dependence ln X as a function of time is expected to take the form of a straight line whose slope is μ_{net}. By changing the initial concentration of the growth substrate in the solution $\mu_{net} = f(S_{l0})$ data base can be obtained making it possible to select the form and estimation of the kinetic equation constants.

In the processes of biological purification of air or sewage the key parameter, which is the measure of the process efficiency, is the rate of the utilization of pollutant. Hence, it is necessary to know the parameter enabling the transformation of the equation describing the rate of biomass growth to the equation describing the rate of utilization of growth substrate.

The observed biomass yield coefficient $(Y_{xs})_{obs}$ is such a parameter, and it is defined as the ratio of the rate of biomass growth to the rate of utilization of the substrate, measured in the same time interval of growth phase.

The substrate mass loss as a result of biodegradation, can be connected with the biomass growth via the following relationship:

$$\frac{dm_s}{dt} = -\frac{V_l}{(Y_{xs})_{obs}} = (V_l)\frac{dS_l}{dt} \tag{3}$$

where m_s [g] is the total mass of growth substrate in liquid phase, V_l [m^3] is volume of liquid phase in biostat, S_l [$g \cdot m^{-3}$] is phenol concentration in liquid phase.

Integrating dependence (3) over the time interval of the test we obtain:

$$X = X_0 + (Y_{xs})_{obs}(S_{l0} - S_l) \tag{4}$$

where

$$S_{l0} = \frac{m_{s0}}{V_l} \tag{5}$$

and m_{s0} is the mass of phenol initially introduced into the biostat. Equation (4) can be rearranged to the form, which allows calculating the observed yield coefficient as follows:

$$(Y_{xs})_{obs} = \frac{(X - X_0)}{(S_{l0} - S_l)} \tag{6}$$

The reason for the termination of growth may be exhaustion of the essential nutrients. Therefore, when the growth substrate is depleted, the endogenous decay of the culture begins. The appropriate equation to describe the loss of cell mass due to cell lysis is:

$$\frac{dX}{dt} = -k_d \cdot X \tag{7}$$

where k_d is a first-order rate constant for endogenous decay. Integrating equation (7) we obtain:

$$\ln X = -k_d t + \ln X_{d0} \tag{8}$$

where X_{d0} is the concentration of cells at the beginning of the decay phase. A plot of ln X versus t yield a line of slope ($-k_d$). It must be emphasized that the endogenous decay coefficient k_d is a parameter of models describing cometabolic biodegradation of various compounds in the presence of phenol as a growth substrate. Therefore, it is necessary to determine the value of this parameter for future applications.

Materials and methods

Stenotrophomonas maltophilia KB2 strain, coming from collection of Department of Biochemistry, Faculty of Biology and Environment Protection, University of Silesia, was originally isolated from the active sludge of sewage treatment plant.

The adaption of *Stenotrophomonas maltophilia* KB2 strain to use phenol as the only carbon and energy source was carried out in 500 cm^3 Erlenmeyer flasks, in an incubatory shaker, at 30°C. The composition of mineral medium was described previously [22].

2.25 cm^3 of phenol solution (400 mmol) was added to the culture every 24 h and the optical density of bacterial suspension was measured every day. After 5 days of cultivation, the cell suspension was centrifuged (5000 rpm for 10 minutes) and stored at 4ºC.

The optical density (OD) method was used to measure cell concentration. OD was determined using spectrophotometer HACH DR3900 (HACH-Lange, Germany) at λ = 550 nm against distilled water as a reference. To determine cell concentration by absorbance measurement the calibration curve between optical density of the bacterial suspension versus dry cell mass was prepared previously.

Phenol concentration in liquid samples was determined by HPLC method using Waters chromatograph equipped with UV/VIS detector and silica-based, reversed-phase column (Spherisorb ODS2, 150 × 4.6 mm, 5 µm). Mixture of methanol and 1% acetic acid in water was used as a mobile phase (v:v 40:60). Detection was carried out at wavelength of 272 nm. The liquid samples were taken from bioreactor and centrifuged (9000 rpm) for 10 minutes. Supernatant was filtered through syringe filters (PTFE membrane, pore size 20 µm) and diluted with water (1:1). Before proceeding with the research associated with the determination of kinetic model the effect of temperature and pH on the growth of the tested strain and phenol degradation were examined. In this way the optimal environmental conditions for the process were determined.

Batch experiments were carried out in Biostat B fermentor (Sartorius, USA) with working volume of 2.7 dm^3. During experiments the optimal environmental conditions were maintained (30ºC and pH 7) and stirrer rotations 300 rpm. The air was supplied by external compressor through a sterilized filter and concentration of dissolved oxygen (DO) was about 5 mg·dm^{-3}. Each cultivation was started with the similar cells concentration in the solution, equaling 61.3 g_{dcw}·m^{-3} (the initial OD about 0.2 at λ = 500 nm). The culture was sampled at regular time intervals and both absorbance and phenol concentration were determined.

Results

Effect of temperature

To determine the influence of temperature on *Stenotrophomonas maltophilia* KB2 growth and phenol degradation a series of experiments at different temperatures (range 22-42ºC) and at pH 7 were performed. First part of tests was carried out in Erlenmeyer flasks containing 300 cm^3 of microbial culture, with 2.25 cm^3 of phenol solution added every 24 h. The growth of tested strain was observed for temperatures changed within the range 22-38ºC. *Stenotrophomonas maltophilia* KB2 incubated at 42ºC did show neither growth nor phenol degradation and at 22ºC the growth of biomass was less intensive than for other temperatures. Taking it into account, the second part of the tests was carried out in batch reactor for temperatures changed within the range 22-38ºC and initial concentration of phenol equaled 75 g·m^{-3}. Figures 1a and 1b show the results of experiments. It could be observed that both the specific growth rate of KB2 strain and the rate of phenol biodegradation were most effective for temperatures equaling 30 and 34ºC. Similar range of temperatures was found by Wojcieszynska et al. [21] as an optimum for activity of monooxygenase isolated from KB2 strain. Therefore, the kinetic experiments were conducted at 30ºC.

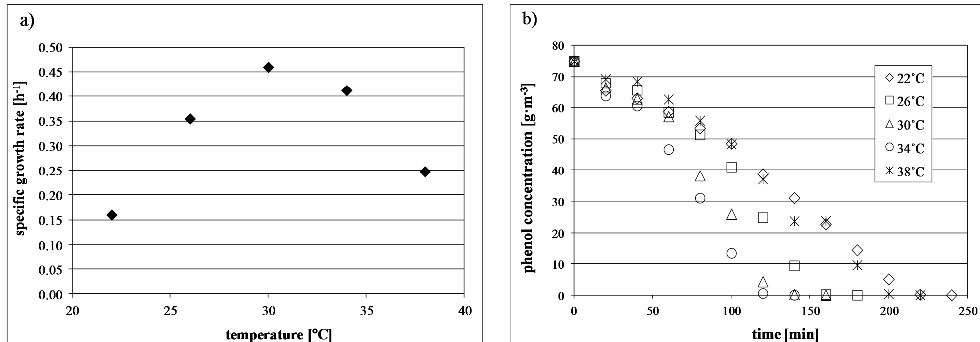

Fig. 1. The rate of growth KB2 strain (a) and phenol biodegradation at different temperatures (b)

Effect of pH

The value of pH solution may affect the rate of biochemical reaction [23]. Therefore, the first series of experiments was carried out in Erlenmeyer flasks, at 30ºC and initial phenol concentration equaled 75 $g\cdot m^{-3}$, changing pH in the range of 4.5-9. The tests showed the lack of growth KB2 strain for the values of 4.5 and 9. Another series of experiments was performed in batch reactor for initial phenol concentration in the solution equaling 75 $g\cdot m^{-3}$ and for the range of changes of pH 5-8. It was found that specific growth rate of microorganisms increases for pH changed within the range of 5-6 and decreases for pH above 7. There were no significant differences in the rate of phenol utilization for pH of 6-7 (Fig. 2).

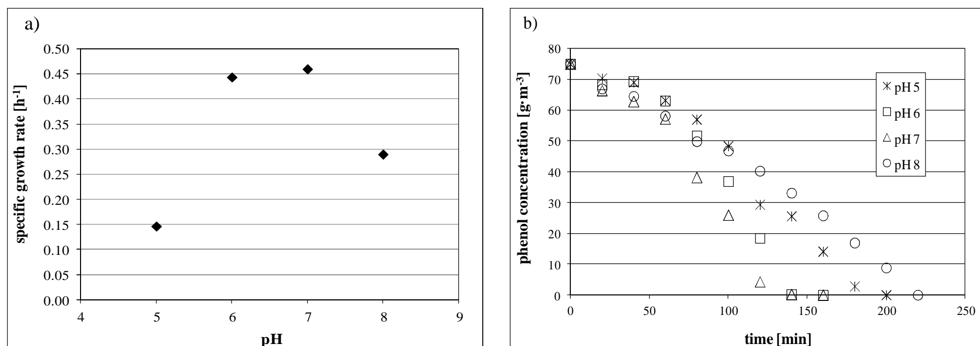

Fig. 2. Specific growth rate of KB2 strain (a) and changes in phenol concentration (b) in the cultures carried out at different pH values, $t = 30$ºC

Additionally, for each tested pH value a series of experiments was carried out using uninocculated culture to check whether the decrease in phenol concentration is a result of chemical reaction or not. The results showed that after a few hours of experiments there was no substrate loss. Therefore, pH had no effect on the concentration of phenol in the uninocculated samples.

Wojcieszynska et al. [21], investigating activity of enzymes taking part in phenol decomposition, stated that the highest activity of phenol monooxygenase isolated from KB2 strain was observed for similar value of pH, equaling 7.2. Taking above into account, the kinetic tests were performed for pH solution equaling 7.

Effect of initial cell concentration

The series of batch cultures was performed for initial cell concentrations changed in the range of 23.0-92.0 $g_{dcw}\cdot m^{-3}$ (absorbance from 0.075 to 0.3) and initial phenol concentration in the solution equal 75 $g\cdot m^{-3}$. It was observed (Fig. 3) that phenol was effectively decomposed for initial biomass concentrations about 61.3-92.0 $g_{dcw}\cdot m^{-3}$. Taking it into consideration the kinetic tests were carried out in batch reactor for initial concentration of biomass equaling 61.3 $g_{dcw}\cdot m^{-3}$.

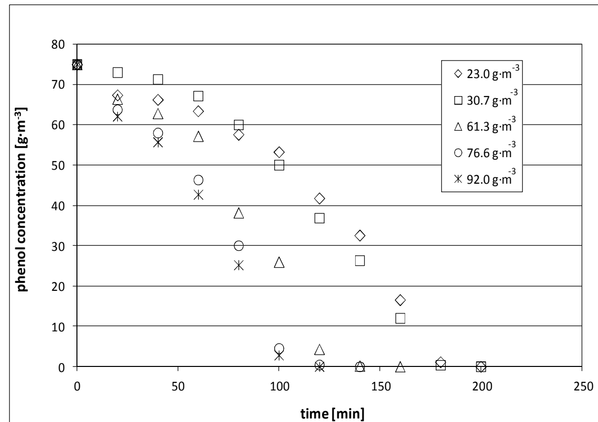

Fig. 3. Phenol biodegradation at different initial biomass concentration [$g\cdot m^{-3}$]

Effect of substrate concentration

The batch cultures of *Stenotrophomonas maltophilia* KB2 strain tests were carried out at 30ºC and pH 7. A constant value of pH was maintained by feeding 10% solution of KOH or KH_2PO_4. Each culture was started at a similar concentration of cells in the suspension (61.3 $g_{dcw}\cdot m^{-3}$) and for initial phenol concentration changed within the range of 25 to 500 $g\cdot m^{-3}$. Figure 4 shows the curves representing the rates of substrate utilization (a) and biomass growth (b) for different initial phenol concentrations. For every experimental point the graph of $\ln X = f(t)$ was plotted; in the exponential growth phase the dependence is a straight line of slope (μ_{net}). The data-base compiled in this way, presented in Figure 5, confirms the earlier information about an inhibitory effect of phenol on the growth of microorganisms.

A lot of propositions of equations describing kinetics of microorganisms growth inhibited by substrate can be found in literature [24]. The most frequently applied the Haldane equation was chosen to describe the *Stenotrophomonas maltophilia* KB2 strain growth on phenol:

$$\mu = \frac{(\mu_m \cdot S)}{K_s + S + \dfrac{S^2}{K_i}} \tag{9}$$

where μ_m [h^{-1}] is the model parameter, K_s and K_i are the half saturation constant [$g \cdot m^{-3}$] and substrate inhibition constant [$g \cdot m^{-3}$], respectively.

The kinetic equation parameters were estimated basing on the own database using the least - square error method with the help of NLREG programme (Sherrod 2010), and they are: $\mu_m = 0.9\ h^{-1}$, $K_s = 48.97\ g \cdot m^{-3}$, $K_i = 256.12\ g \cdot m^{-3}$.

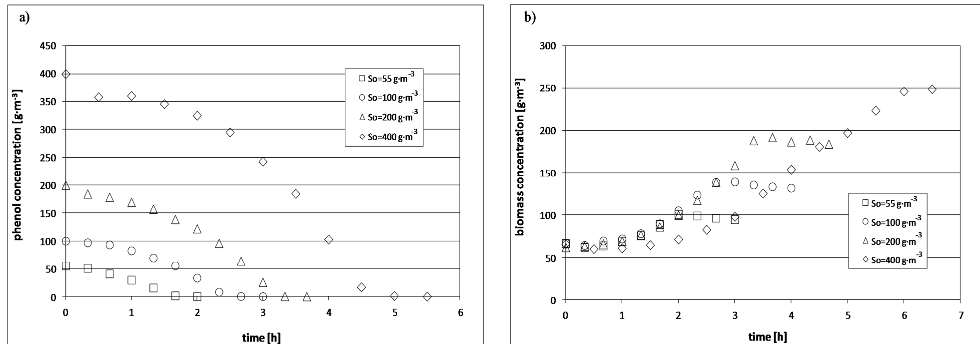

Fig. 4. Changes in phenol (a) and biomass (b) concentrations versus time for different initial substrate concentrations

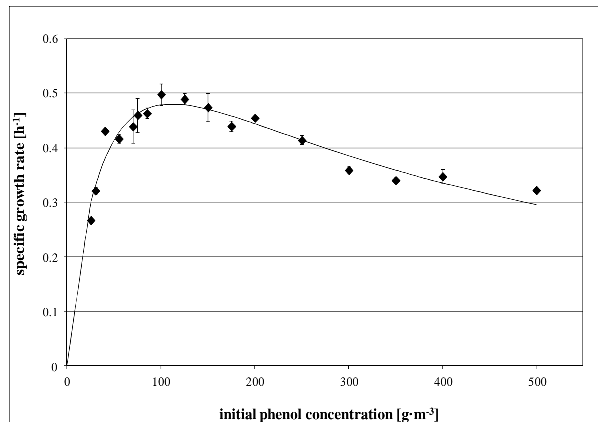

Fig. 5. Effect of initial phenol concentration on specific growth rate of KB2 strain

Developed equation with a mean relative error:

$$e_Y = \frac{1}{N} \sum_{i=1}^{N} \left| \frac{\mu_{exp,i} - \mu_{calc,i}}{\mu_{exp,i}} \right| \cdot 100\% \tag{10}$$

not exceeding 5% approximates the experimental data. In Equation (10) subscripts *exp* and *calc* denote experimental and calculated values, respectively.

The values of kinetic parameters and growth conditions were compared with literature data in Table 1 and Figure 6. The comparison of these data reveals that the specific growth rate for *Stenotrophomonas maltophilia* KB2 strain was one of the highest. The tested strain showed also great resistance to inhibition of their growth by phenol (high value of substrate inhibition constant), similar to the values obtained for *Pseudomonas putida* ATCC 49451 [25], *Bacillus cereus* [2] and *Alcaligenes faecalis* [15].

Table 1

Microorganisms, the values of kinetic parameters and culture conditions for phenol biodegradation in different studies

Bacterial strain	**Phenol concentration range [g·m^{-3}]**	**Haldane model parameters**			**$(Y_{XS})_{obs}$ [$g_x \cdot g_s^{-1}$]**	**Culture condition**		**References**
		μ_m [h^{-1}]	**K_s [g·m^{-3}]**	**K_i [g·m^{-3}]**		**Temp. [°C]**	**pH [-]**	
Bacillus cereus	50-600	0.153	21.33	238.0	0.102-0.880	30	7.0	[2]
Pseudomonas putida MTCC 1194	10-1000	0.305	36.33	129.79	0.65	30	7.1	[4]
Pseudomonas putida DSM 548	1-100	0.436	6.19	54.1	0.0017	26	6.8-6.3	[8]
Alcaligenes faecalis	0-1800	0.15	2.22	245.37	–	–	–	[15]
Pseudomonas sp. cbp 1-3	0-1400	0.275	6.9	530.7	–	30	7.2	[17]
Pseudomonas putida ATCC 49451	100-500	0.9	6.93	284.3	–	30	6.5	[25]
Pseudomonas putida LY1	20-800	0.217	24.4	121.7	0.765 (for concentration 50 g·m^{-3})	25	7.1	[26]
Pseudomanas putida (Tan1) *Staphylococcus ureus* (Tan2)	100-800	3.56	18.7	212.48	0.4258	35	6.0	[27]
Mixed cultures	25-1450	0.143	87.44	107.06	0.6	25	6.9	[28]
Stenotrophomonas maltophilia strain KB2	25-500	0.9	48.97	256.12	0.65	30	7.0	this study

The mean value of the biomass yield coefficient, determined in the experiments, was $(Y_{xs})_{obs} = 0.614$.

The endogenous decay coefficient (k_d) was obtained by monitoring the decay of cell mass in the absence of growth substrate. The semilog plot of biomass concentration vs. time yields a straight line with slope of ($-k_d$). For *Stenotrophomonas maltophilia* KB2 strain the endogenous decay coefficient evaluated in this way equaled 0.05 h^{-1}.

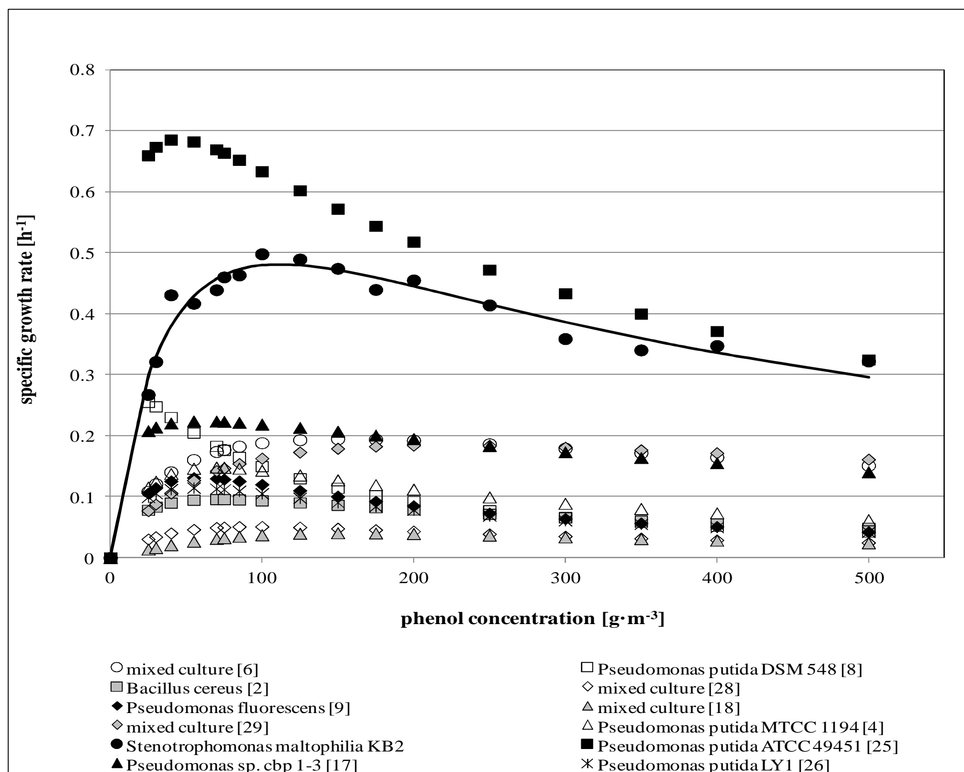

Fig. 6. Comparison of the specific growth rate of different microbial strain

Conclusions

The experiments showed high activity of the tested microorganisms in the process of phenol biodegradation and relatively low sensitivity to the inhibiting influence of growth substrate at higher concentrations in solution. A large experimental data base made it possible to estimate the constants of kinetic equation, to determine the yield coefficient and the endogenous decay coefficient.

The studies showed the great potential of *Stenotrophomonas maltophilia* KB2 strain to use phenol as a carbon and energy source. Due to such feature, the examined microorganisms may be recommended as biological material in the industrial processes of removing phenol from wastewater.

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MODELOWANIE TLENOWEJ BIODEGRADACJI FENOLU PRZEZ SZCZEP *Stenotrophomonas maltophilia* KB2

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Abstrakt: Przedmiotem badań była kinetyka biodegradacji fenolu przez bakterie *Stenotrophomonas maltophilia* KB2. Szczep ten został wyizolowany z osadu czynnego oczyszczalni ścieków komunalnych w Bytomiu-Miechowicach i jest obecnie przechowywany w kolekcji VTT (Finlandia) pod numerem E-113137. Eksperymenty kinetyczne prowadzono w reaktorze okresowym, zmieniając w kolejnych eksperymentach stężenie fenolu, który był dla testowanego szczepu jedynym źródłem węgla i energii, w zakresie 25-500 $g \cdot m^{-3}$. Testy kinetyczne poprzedzone zostały serią hodowli prowadzonych w zmiennych warunkach środowiskowych ($5 \leq pH \geq 8$; $22°C \leq t \geq 42°C$; początkowe stężenie biomasy 22-92 $g_{dcw} \cdot m^{-3}$) w celu określenia warunków hodowli najkorzystniejszych dla wzrostu stosowanych mikroorganizmów. Były to: pH 7, t = 30ºC, początkowe stężenie biomasy ~61,3 $g_{dcw} \cdot m^{-3}$ i przy takich wartościach testowanych parametrów przeprowadzono wszystkie eksperymenty kinetyczne. Testy kinetyczne wykazały, że dla początkowych stężeń fenolu w roztworze większych od ~100 $g \cdot m^{-3}$ zaczyna być widoczny inhibitujący wpływ substratu na wzrost mikroorganizmów. Zatem, do opisu kinetyki wzrostu szczepu KB2 na fenolu wybrano model Haldane. Parametry tego równania wyestymowano w oparciu o własną bazę danych eksperymentalnych. Są to: μ_m = 0,9 h^{-1}; K_s = 48,97 $g \cdot m^{-3}$, K_i = 256,12 $g \cdot m^{-3}$. Opracowane równanie kinetyczne przybliża dane eksperymentalne ze średnim błędem procentowym

nieprzekraczającym 5% (R^2 = 0,95). Przeprowadzone testy pozwoliły również na wyznaczenie średniej wartości współczynnika wydajności biomasy ($(Y_{xs})_{obs}$ = 0,614), który umożliwia transformację zależności opisującej szybkość wzrostu mikroorganizmów w równanie opisujące szybkość biodegradacji fenolu. Wyznaczony został również współczynnik endogennego zamierania, k_d = 0,05 h^{-1}, którego wartość jest niezbędna przy modelowaniu układów kometabolicznych, w których fenol jest substratem wzrostowym. Badania wykazały, że szczep *Stenotrophomonas maltophilia* KB2 jest dobrym materiałem do zastosowań przemysłowych (krótka faza zastoju, duża aktywność biodegradacyjna, optymalna szybkość wzrostu osiągana przy stosunkowo dużych stężeniach fenolu w roztworze).

Słowa kluczowe: fenol, biodegradacja, hodowla okresowa, równanie kinetyczne