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KINETICS OF THE BIODEGRADATION OF MONOAROMATICS BY *Pseudomonas aeruginosa*

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Abstract - Water contamination by monoaromatic compounds has risen throughout time, which leads to the necessity of developing new water treatment technology, capable of minimizing their negative effect on the environment. In this context, biological processes present themselves as a solution to the processes of extraction. Bioremediation makes use of microbial groups capable of using hydrocarbons as a source of carbon to perform their metabolic functions. This work evaluated the biodegradation efficiency of *Pseudomonas aeruginosa* strain isolated from contaminated matrices, for the substrates benzene, ethylbenzene and toluene, aiming to determine to which compound the bacteria had better adaptation. For that, bioremediation assays were performed for each of the monoaromatic compounds, in an isolated way, with the goal of obtaining experimental data and from this Monod and Andrews kinetic models were discretized and numerically developed through the Runge-Kutta method. It was possible to observe that *Pseudomonas aeruginosa* has a bigger affinity for ethylbenzene, while benzene generated a bigger microbian coefficient. Monod´s model was capable of predicting satisfactorily the experimental data.
Keywords: Water treatment; Monoaromatic compounds; *Pseudomonas aeruginosa*; Bioremediation; Biological processes.

INTRODUCTION

Monoaromatic compounds, such as benzene, toluene and ethylbenzene, which have their chemical structures shown in Figure 1, represent an important class of pollutant due to their high toxic potential to different organisms (Anneser et al., 2008) (JO et al., 2008). They are found in oil derivatives and are widely used in chemical industries as raw-materials for the synthesis of other products (Phelps and Young, 2001).

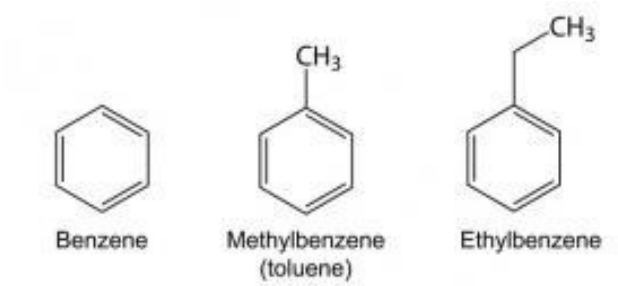

Figure 1. Monoaromatic compounds chemical structure (Benzene, toluene and ethylbenzene).

Environmental pollution caused by petroleum hydrocarbons represents a great risk to ecosystems (Mousa et al., 2014). In a specific way, monoaromatic compounds present an elevated solubility in water, which facilitates the migration and fast contamination of underground water and soil by these compounds that, even in low concentrations, may cause serious harm to the environment and to human health (Aivalioti et al., 2012) (Mazzeo et al., 2010) (Jo et al., 2008) (Santaella et al., 2009) (Paixâo et al., 2007).

The high mobility of these hydrocarbons in soil-water systems is related to their low coefficient of partition octanol-water, which gives the soil a low absorption potential, and, consequently, a preferred

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transport through water. This fact favors the contamination of water reservoirs (Nakhla, 2003).

As the contamination of underground water by hydrocarbons has been growing (REUSSER et al., 2002) there is the necessity of developing more efficient methods to remove or minimize the damage caused by these compounds (Mazzeo et al., 2010).

However, normally, the conventional physical-chemical treatments used to remove monoaromatic compounds from the environment, besides demanding high operational costs, do not destroy the contaminants, only phase transport them. In that sense, biological processes are considered an efficient technology, especially because of their operational simplicity and low cost when compared to other methods (Moussa et al., 2014) (Bertin et al., 2007) (Massalha et al., 2007) (Mazzeo et al., 2010).

Bioremediation consists of using microbian groups capable of degrading hydrocarbons. These microorganisms are able to biotransform pollutant molecules into nutrients for the realization of their metabolic and physiologic functions. The biological degradation processes (biodegradation) of the organic compounds are made through the breaking of these compound into less toxic substances, such as CO_2, water and methane (Bittkau et al., 2004) (Farhadian et al., 2009) (Martínez et al., 2007).

Degradations of aromatic compounds using bacteria of the *Pseudomonas* gender are commonly mentioned, such as *Pseudomonas putida* (Ridgway et al., 1990; Lee et al., 1994; Otenio et al., 2005; Shim et al., 2005; Lin et al., 2010); *Pseudomonas aeruginosa* (Ridgway et al., 1990;, Nwinyi et al., 2016; Chebbi et al., 2017; Khodaei et al., 2017); *Pseudomonas fluorescens* (Ridgway et al., 1990; Shim et al., 2005; Oberoi and Philip, 2017); *Pseudomonas aureofaciens* (Dou et al. 2008); *Pseudomonas stutzeri* (Singh and Tiwary, 2017); *Pseudomonas plecoglossicida* (Li et al., 2017).

This work used as microorganism *Pseudomonas aeruginosa,* a Gram-negative bacterium, which can be isolated from different habitats including water, the soil and the plants, and under specific environmental conditions. Besides that, it is able to produce a biosurfactant containing the glycolipid ramnose. The type and the proportion of the rhamnolipid produced depends on the strain, of the carbon source used and of the cultivation conditions (Santa Anna et al., 2002; Fontes et al., 2008). Joined to this capability, *Pseudomonas aeruginosa* consumes monoaromatic compounds, using them as a source of carbon and energy, besides presenting high growth rates under high substract concentrations (Malhautier *et al*., 2014).

The knowledge of the biodegradation kinetics of these pollutants and their adjustment to a model is indispensable for the project of adequate treatment systems on a real scale. Therefore, this study aims to investigate and model the biodegradation of the monoaromatic compounds benzene, toluene and ethylbenzene in water and the evaluation of their consumption by the bacteria *Pseudomonas aeruginosa* over time, using a greater compound concentration range than the one commonly used in previous studies.

METHODS

Pseudomonas aeruginosa

A bacterial strain of *Pseudomonas aeruginosa* was selected from a soil that was artificially contaminated with a solution of diesel oil of 2.50 g/l and moisturized with the mineral medium described by Robert et al. (1989), in order to provide all the nutrients needed for the development of the microorganism. This strain was isolated and selected through inoculation in Acetamide Agar qualitative medium and Cetrimide Agar quantitative medium, which are both selective media for *Pseudomonas aeruginosa.*

After a pH adjustment to 7.0, the cultivation medium was sterilized at 121°C and 1 atm during 15 minutes. As a pre-inoculum, a nutrient solution of peptone, 2% in mass, was used, which remained in the shaker at 200 rpm agitation and at 30 °C for 10 hours (Miguez et al., 2012).

Bioremediation essays

In the bioremediation assays 50 mL of the medium described by Robert et al. (1989) was used, in erlenmeyers of 125 mL, inoculated with 1 mL of the pre-inoculum (cellular suspension of *Pseudomonas aeruginosa*) and contaminated synthetically from a concentrated solution of benzene, ethylbenzene or toluene in initial concentrations of 200 mg/L, 150 mg/L and 170 mg/L, respectively, that were defined from the solubility of them in the water medium.

The erlenmeyers were accommodated in a shaking table, at 28 degrees Celsius and 200 rpm, kept for 3 hours, with samples taken each 15 minutes, for celular growth e substrate consumption analysis.

Analytical measurements

The cellular concentration was determined through the dry mass. Therefore, samples of the fermented medium were filtered through a previously weighed membrane, followed by system introduction (membrane+cells) into an incubator at 105 °C. After 2 hours of drying, the membrane was put in a desiccator with silica gel until cooled and, just then the membrane with the dry cellular mass was weighed. The mass determination was made by the difference of the final and initial weight of the membranes. The membranes used for the execution of this analysis were the membranes for ultra-filtering of pore diameter of 0.45 μm and brand MILLIPORE® (Vitolo et al., 1995).

The determination of the substrate consumption was made by liquid chromatography. Therefore, a C18 HPLC column of 15 cms length and 4.6 mm of internal diameter was utilized, with a solution of 70% of acetronitrile and 30% of water in volume, in a flow of 1 mL/min, and injecting 50 microliters of the sample. Prior to sample reading a standard curve was made for each compound, using a range from 10 mg/L up to the maximum solubility in water concentration.

Modeling

The modeling of the biodegradation of these compounds enables the study of the influences of the process parameters on the biomass growth and substrate consumption, besides the formation of products, being important for the comprehension of microbian biology, as well as internal control mechanisms (Trigueros et al., 2010).

The Monod model (Equation 1) is applied to study the kinetics of the biodegradation of a single substrate. In it, the parameters are the specific growth rate (μ_{max}) and the Monod (K_S) saturation constant, which represents the value of concentration of the substrate S, at which the specific growth rate is equal to half of its maximum value, and indicates especially the affinity of the microorganism and the substrate.

Monod considers that all the components of the culture medium, with the exception of the substrate (monoaromatic compounds), are present in high concentrations, in such a way as changes in these conditions do not affect significantly the cellular growth rate (Bailey and Ollis, 1986).

$$\mu_x = \mu_{max} \frac{S(t)}{K_S + S(t)} \tag{1}$$

The Andrews model (Equation 2) considers the substrate limitation through the inhibition constant K_I, that, if too big indicates that the bioprocess inhibition does not occur because of the substrate presence. If $K_I >> S$, the Andrews model becomes the Monod model.

$$\mu_x = \mu_{max} \frac{S(t)}{K_S + S(t) + \frac{S(t)^2}{K_I}} \tag{2}$$

This kinetic model was incorporated into the mass balance for each substrate and biomass in a bioreactor operating in the batch mode (Equations 3 and 4, respectively) where Yx/s corresponds to the substrate tax that is transformed into biomass.

$$\frac{dS(t)}{dt} = -\frac{\mu_x}{Y_{x/s}} \cdot X(t) \tag{3}$$

$$\frac{dX(t)}{dt} = \mu_x \cdot X(t) \tag{4}$$

The differential equations mentioned were discretized and the tangent function was adjusted through the Runge-Kutta 4th order method. The evaluation of the model efficiency was made through the minimum value of the objective function (F. O.), given by the minimum squares. To the variables substrate (*S*) biomass (*X*), the calculus of the objective function is given by the Equations 5 and 6, respectively.

$$F.O. = \sum_i \left(\frac{S_i^{cal}}{S^{max}} - \frac{S_i}{S_{max}} \right)^2 \tag{5}$$

$$F.O. = \sum_i \left(\frac{X_i^{cal}}{X^{max}} - \frac{X_i}{X_{max}} \right)^2 \tag{6}$$

For each substrate the relative error sum (e_m) of all the points was calculated using Equation 7, that represents the difference between the sum of the experimental values (X) and the values obtained through the model (X_i^{cal}), over the experimental values.

$$e_m = \frac{\sum_i^n \left(\frac{X_i - X_i^{cal}}{X_i} \right)}{n} + \frac{\sum_i^n \left(\frac{S_i - S_i^{cal}}{S_i} \right)}{n} \tag{7}$$

RESULTS AND DISCUSSION

The results of the consumption of toluene, ethylbenzene and benzene are presented in Figures 2, 3 and 4, respectively. After 2 hours, for most initial concentrations that have been studied, the

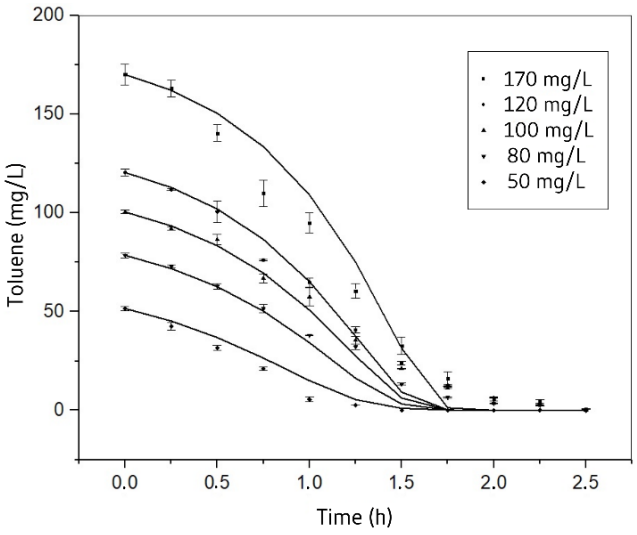

Figure 2. Experimental (symbol) and calculated data (lines) of toluene consumption with time.

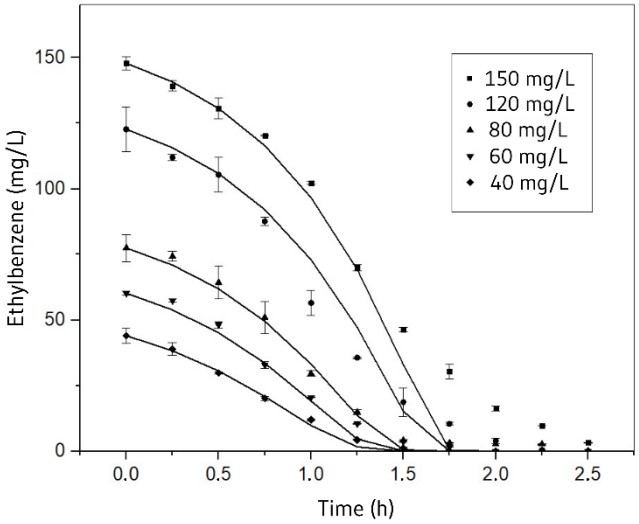

Figure 3. Experimental (symbol) and calculated data (lines) of ethylbenzene consumption with time.

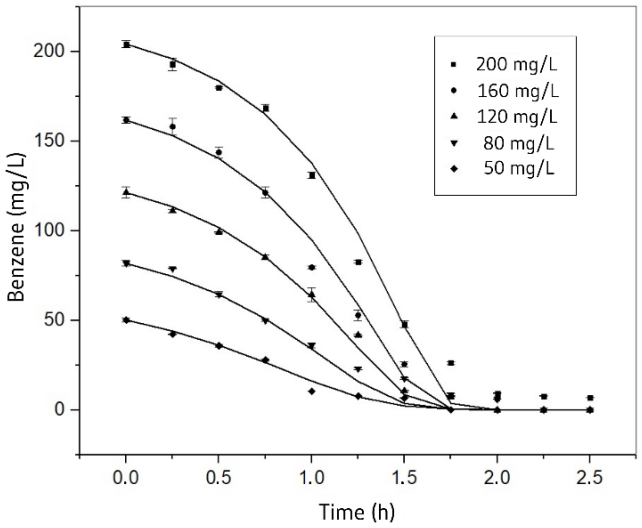

Figure 4. Experimental (symbol) and calculated data (lines) of benzene consumption with time.

concentrations of toluene, ethylbenzene, and benzene reached zero and the biodegradation yield reached 100% after 2.5 hours of test. These values are more expressive than previous studies with other microorganisms., that obtained yields near 80 % with a kinetic time varying from 6 to 48 hours (Khodaei et al., 2017; Nagarajan and Loh, 2014; Drakou *et al.*, 2015; El-Naas et al., 2014 Morlett-Chávez et al., 2010; Parameswapara et al., 2008).

The biomass growth curves for toluene, ethylbenzene and benzene are presented in Figures 5, 6 e 7 respectively, where it was possible to verify that none presented a lag phase of growth, which shows that a period of adaptation to the substrate was not necessary. This can be proved plotting the curve of lnX versus time, which indicated the beginning of the exponential phase of growth during the first instants of cultivation. It was also possible to observe that,

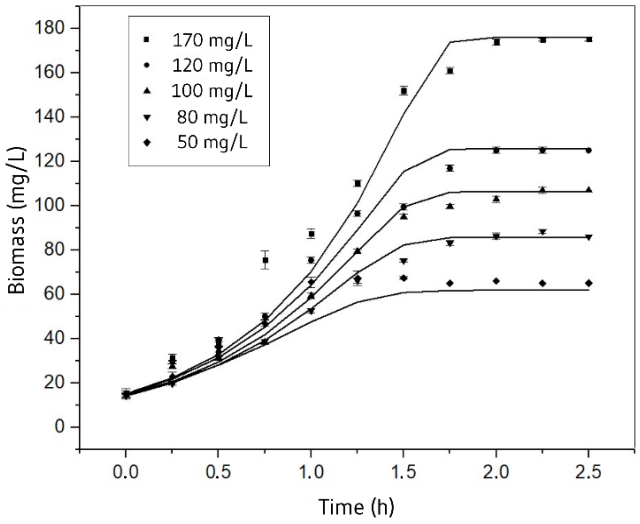

Figure 5. Experimental (symbol) and calculated data (lines) biomass growth for toluene.

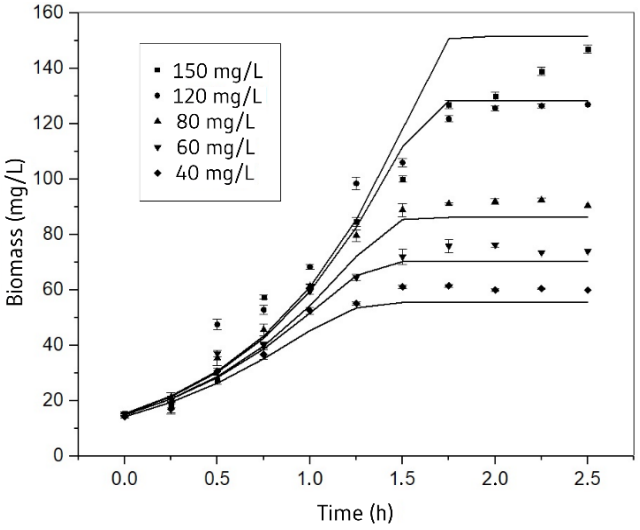

Figure 6. Experimental (symbol) and calculated data (lines) biomass growth for ethylbenzene.

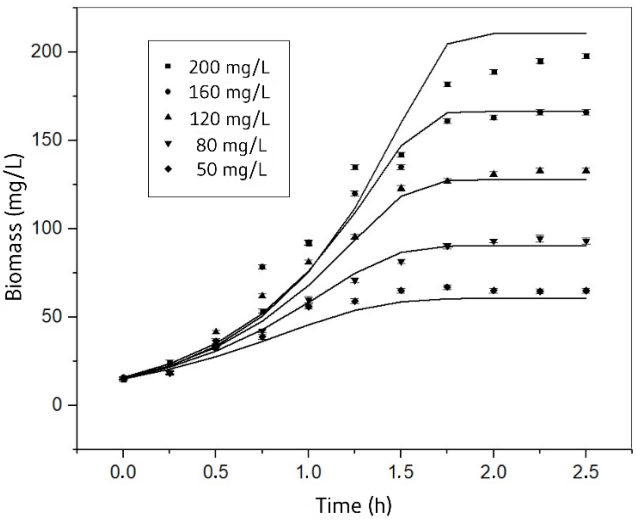

Figure 7. Experimental (symbol) and calculated data (lines) biomass growth the benzene.

with carbon availability reduction, a reduction in the growth rate of *Pseudomonas aeruginosa* also occurred as shown in other studies (Drakou *et al.*, 2015; Malhautier *et al*., 2014).

The modeling of the experimental data which refer to the biomass generation and substrate consumption curves of toluene, ethylbenzene and benzene made from the differential equations 3 and 4 corresponds to the solid curves of Figures 2 to 7. The kinetic behavior of the three substrates is consistent with the Monod model with an average error varying between 0.194 to 0.465, while there was no adjustment to the Andrews model, showing that the chemical biodegradation of the studied monoaromatics does not suffer inhibition from the substrate. The parameter values of the Monod model for each one of the substrates are presented in Table 1, including the values of the average errors of the Monod model for each monoaromatic.

The value of μ_{max} is compatible with other studies made for different types of *Pseudomonas.* For toluene, they obtained 0.053 h^{-1} with a mixed microbial consortium (Rajamanickam et al., 2017), 0.17 h^{-1} for *Pseudomonas putita* (Mathur and Majumder, 2010), 0.42 h^{-1} for *Pseudomonas putita* 54g (Mirpuri et al., 1997), 1.56 h^{-1} for *Pseudomonas putita* OI (Oh et al., 1994) and 0.78 h^{-1} for *Pseudomonas putita* F1 (Bordel et al., 2007). As for benzene: 0.1613 h^{-1} for *Pseudomonas putita* (MA/athur and Majumder, 2010), 0.5 h^{-1} for *Pseudomonas putida* F1 (Robledo-Ortíz et al., 2011) and 0.75 h^{-1} for *Pseudomonas putida* F1 (Abuhamed et al., 2004).

The maximum specific growth rate obtained for each monoaromatic showed a tendency of reduction upon substitution of the aromatic ring with the alkyl group, with the bigger chain of the substituent group, the lower the rate. This result is consistent with those obtained from the kinetics of toluene in contrast with benzene with *Pseudomonas putida* (Mathur and Majumder, 2010) and *Pseudomonas putida* F1 (Abuhamed et al., 2004).

The saturation constant (*Ks*), which is defined as the substrate concentration in which μ_x is equal to half of μ_{max} also presented values on the same order of magnitude of those presented in the literature. For toluene, 62.56 mg/L was obtained for *Pseudomonas putita* (Mathur and Majumder, 2010), 3.98 mg/L for *Pseudomonas putita* 54G (Mirpuri et al., 1997), 15.07 mg/L for *Pseudomonas putita* OI (Oh et al., 1994) and 5.00 mg/L for *Pseudomonas putita* F1 (Bordel et al., 2007). As for benzene: 71.18 mg/L for *Pseudomonas putita* (Mathur and Majumder, 2010), 10.11 mg/L for *Pseudomonas putida* F1 (Robledo-Ortíz et al., 2011) and 1.65 mg/L for *Pseudomonas putida* F1 (Aabuhamed et al., 2004).

Table 1. Kinetic parameters.

Parameter	Compound		
	Benzene	Toluene	Ethylbenzene
$\mu_{máx}$ (h^{-1})	1.86	1.72	1.48
K_s (mg/L)	22.67	16.53	7.53
$Y_{x/s}$ (mg/mg)	0.848	0.849	0.851
e_m	0.364	0.194	0.465

The lowest Ks value was obtained for ethylbenzene, indicating that the microorganism presents a bigger compatibility with this compound. This can be explained by the fact that groups in the aromatic ring affect its reactivity. The presence of alkyl groups activates the ring, making it more reactive and easier to break. The bigger the alkyl group chain is, the more electrical density is donated and more reactive is the aromatic ring, which explains the increased compatibility of ethylbenzene in relation to the other compounds that were studied. These results are consistent with the ones obtained through other studies (Littlejohns and Daugulis, 2008; Trigueros et al., 2010).

Some investigations have considered benzene to be a recalcitrant compound in anaerobic conditions. This behavior is generally explained due to the stabilization of the carbon-carbon bonds and the symmetric structure of the ring that make the benzene highly resistant to breaking, besides other factors (Trigueros et al., 2010).

It has also been observed that *Pseudomonas aeruginosa* presented a good mineralization rate, because the values of $Y_{x/s}$, the yield of substrate transformed into biomass, were higher than the ones presented in literature for *Pseudomonas putida* F1. For benzene: 0.60 (Robledo-Ortíz et al., 2011), 0.75 (Abuhamed et al., 2004) and for toluene: 0.60 (Robledo-Ortíz et al., 2011), 0.58 (Abuhamed et al., 2004).

The modeling of the experimental data by the equations of Monod has been shown to be efficient, confirming that in the case of the three observed substrates their presence does not affect the cellular growth rate. This shows that the presence of the substrate does not inhibit the process. This was also proved by the lack of adjustment to the Andrews model, which through the K constant predicts inhibition by the substrate. These results are similar to the ones obtained in the study of the bioremediation in systems using a single substrate (Reardon et al., 2000; Bielefeldt and Stensel, 1999).

CONCLUSIONS

The kinetics of the biodegradation of monoaromatic compounds (benzene, ethylbenzene and toluene) by *Pseudomonas aeruginosa* suggest a promising strategy for the cleansing of monoaromatic contaminated waters, since the strain that was used did not present difficulties in totally degrading the aromatic ring.

Furthermore, the study of the kinetic parameters of the Monod model can be considered an efficient

prediction of the kinetics of biodegradation of such compounds. A greater compatibility of *Pseudomonas aeruginosa* for ethylbenzene was observed, due to the low value of the saturation constant (K_S). Its high mineralization rate is related to the increased reactivity of the aromatic ring caused by the presence of alkyl groups. However, a higher consumption rate was obtained for benzene since it presented the largest specific growth rate.

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