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Inhibition of the acetoclastic methanogenic activity by disinfectants used in the washing of pharmaceutical industry equipment

Inhibición de la actividad metanogénica acetoclástica para diferentes desinfectantes usados en el lavado de equipo en la industria farmacéutica

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Abstract

The pharmaceutical industry uses different types of disinfectants in the washing processes of different equipment, which become part of the wastewaters generated in the production processes. The nature of these compounds can diminish the biodegradability of the effluents; hence, this is an important issue to consider during the design and operation of treatment processes. This study establishes the kinetic parameters for three commercial disinfectants, using anaerobic sludges as inoculum. The doses for the active principle (quaternary salt) that yield a 50% reduction of the specific methanogenic activity of the inoculum were 1.6×10^{-3} kg $\cdot$ m^{-3} for benzalkonium, 8.4×10^{-3} kg $\cdot$ m^{-3} for bactium, and 154×10^{-3} kg $\cdot$ m^{-3} for anibac. The three disinfectants induced a mixed-type inhibition on the anaerobic biodegradability of sodium acetate, the main precursor of methanogenesis during anaerobic digestion.

Keywords: anaerobic digestion, disinfectants, acetoclastic methanogenic, inhibition constants.

Resumen

La industria farmacéutica utiliza en los procesos de lavado de equipo diferentes tipos de desinfectantes que forman parte de las aguas residuales generadas en los procesos de producción. La naturaleza de estos compuestos puede provocar una disminución en la biodegradabilidad de los efluentes, por lo que determinarla es importante para el diseño y operación de los procesos de tratamiento. En este estudio se establecen los parámetros cinéticos para tres desinfectantes comerciales, usando lodos anaerobios como inóculo. Las dosis para el principio activo (sal cuaternaria) para la que se observa una reducción del 50 % de la actividad metanogénica del inóculo fueron 1.6×10^{-3} kg $\cdot$ m^{-3} para benzalconio, 8.4×10^{-3} kg $\cdot$ m^{-3} para bactium y 154×10^{-3} kg $\cdot$ m^{-3} para anibac. Se comprobó que los tres desinfectantes provocan una inhibición de tipo mixto sobre la biodegradabilidad anaerobia de acetato de sodio principal precursor de la metanogénesis en la digestión anaerobia.

Palabras clave: Digestión anaerobia, desinfectantes, metanogénica acetoclástica, constantes de inhibición.

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1 Introduction

Anaerobic digestion (AD) implies the degradation and stabilization of organic matters in the absence of oxygen by microbial consortia and leads to biogas (a mixture of carbon dioxide and methane, a source of renewable energy) and microbial biomass (Chen *et al*., 2008). Many effluents are treated with AD at a large scale; however, AD tends to be inhibited by toxic and inhibitory compounds present in the treated substrate (da Silva *et al*., 2020). The main challenges of the treatment of wastewaters and industrial wastes through AD are still both the detection of the stage at which inhibition occurs (hydrolysis, acidogenesis, acetogenesis, or methanogenesis) and the ulterior identification of the compound causing the inhibition. Ideally, these two steps should be achieved before the treatment to mitigate the problem (Serrano-Meza *et al*., 2020).

The AD is achieved by complex microbial communities. Methane production is performed by methanogenic acetoclastic and hydrogenotrophic microorganisms. In a conventional mesophilic digester, the acetoclastic methanogens of slow growth are responsible for approximately 70% of the produced methane and, in general, are considered the most sensitive to the presence of inhibitors (Moreno-Cruz *et al*., 2018; Astals *et al*., 2015). Therefore, the classical approach to study the inhibitory effect of a specific compound on the biomass activity is to study its effect on acetoclastic methanogens (Meraz, *et al*., 2022, Adriozola, *et al*., 2019).

The pharmaceutical industry produces a large variety of products; therefore, it uses raw materials of diverse chemical nature. It also requires chemical compounds that will ensure a total disinfection of the manufacturing equipment. The wastewaters discharge of a pharmaceutical manufacturing plant does not only convey smell and color that could be unpleasant, but it also affects negatively biological populations. In general, many of the raw materials and auxiliary materials are toxic and usually have high values of chemical oxygen demand (COD) (Hamon *et al*., 2018; Suman and Anjaneyulu, 2005). The wastewaters generated in pharmaceutical plants usually have extreme pH values, very acid or very alkaline; this heterogeneity of the effluents generated by the pharmaceutical industry has led to a limited success of the conventional treatments (biological, physical, and chemical) because these processes are less effective, or even inefficacious, against very stable refractory and toxic compounds.

One of the fundamental auxiliary materials in the pharmaceutical industry is the disinfectant. Its function is to destroy suspended microorganisms and prevent their dissemination. For this reason, it is used in many and diverse conditions, against suspended microorganisms, on the surfaces of inanimate objects; besides, it differs in its activity periods (Echeverry *et al*., 2007). The disinfectant is a type of biocide. Biocides are substances and compounds that contain one or more active substances aimed at destroying, counteract, neutralize, and avoid the action or exert the control on any noxious organism by chemical or biological means. The choosing of an adequate disinfectant depends on the following criteria: their safety and easiness of application, the costs of handling, storage, operation, and infrastructure, as well as the lack or generation of toxic compounds during their use, are key aspects to be taken into account (Domínguez *et al*., 2018, López-Alcantara *et al*., 2022). The most used disinfectant is the one that has quaternary salts as active principle.

The increasing use of anaerobic technology for the treatment of industrial effluents treatment requires the generation of kinetic information for the design and operation of the anaerobic bioreactors. The high complexity within the microbial consortium of the anaerobic digestion makes the process vulnerable to alterations due to the inhibition by the accumulation of long chain fatty acids (LCFA), volatile fatty acids (VFA), free ammonia, and other compounds that can enter the system. The inhibition levels are strongly influenced by the structure and activity of the microbial community. For example, the populations functionally redundant within an anaerobic consortium can prevent inhibition by limiting the accumulation of the intermediaries (LCFA, VFA, ammonia) that cause inhibition (Amha *et al*., 2018; Anwar *et al*., 2021). Therefore, the selection of the inoculum and the temporal adaptation to the inhibitors can prevent failure of the process; but a better understanding of the inhibitory mechanisms is required to accurately predict and prevent the inhibition of the anaerobic process.

The objective of this work was to determine the type of inhibition caused by the three commercial disinfectants: benzalkonium (Benzalkonium chloride at 50%, Tecsiquim SA de CV, Mexico City, Mexico), anibac (Anibac 580 concentrated, at 23.3%, Chemical and cleaning specialties S. A. de C.V., Mexico City, Mexico), and bactium (Bactium 464

disinfectant solution at 0.9%, Baclín Cosmetics S.A. de C.V. Mexico City, Mexico). The three disinfectants have as active principle the N-alkyl dimethyl bencylammonium chloride at different concentrations. Anibac and Bactium also have N-N-dialkyl methylammonium chloride and didecyl-dimethylamonium chloride, respectively.

2 Methodology

2.1 Inoculum

The used inoculum was flocculated anaerobic sludge from an ascending flow anaerobic reactor at laboratory level, fed with lixiviates from fruits and vegetable compost wastes. The inoculum had a concentration of volatile suspended solids (VSS) of 64.7 kg $\cdot$ m^{-3} and a sedimentation speed of 5.3 m$\cdot$ h^{-1}. The initial methanogenic activity of the inoculum was of 0.44 kg COD_{CH4} $\cdot$ kg^{-1} VSS $\cdot$ d^{-1}. The inoculum was re-fed with sodium acetate for its activation to achieve a Specific Methanogenic Activity (SMA) greater than or equal to 1. The initial concentration of acetate was established at 4 g COD L^{-1} in each new feeding.

2.2 Specific methanogenic activity

The Specific Methanogenic Activity was achieved in 120 mL serum bottles. Eighty milliliter of the reduced anaerobic mineral medium (RAMM) (Shelton and Tiedje, 1984) was used for each replicate. Each bottle with 80 mL of RAMM was supplemented with sodium acetate trihydrate to achieve a chemical oxygen demand (COD) of 4 kg $\cdot$ m^{-3} and the needed amount of flocculated anaerobic sludge to reach 1.5 kg VSS $\cdot$ m^{-3}. The pH of the mixture was adjusted to 7.0 and a nitrogen current was used to bubble the mixture. The bottle was then closed with a rubber cap and sealed with an aluminum ring, and immediately incubated at 308.15 K. The assay was left to until 80% of the substrate had been consumed. Biogas was measured daily connecting the bottle to a glass column containing a water solution saturated with sodium chloride at pH 3.5 (Figure 1). The generated methane was calculated by measuring first the volume of the produced biogas, which was determined by multiplying the displacement of the solution in the column by the area of the glass tube.

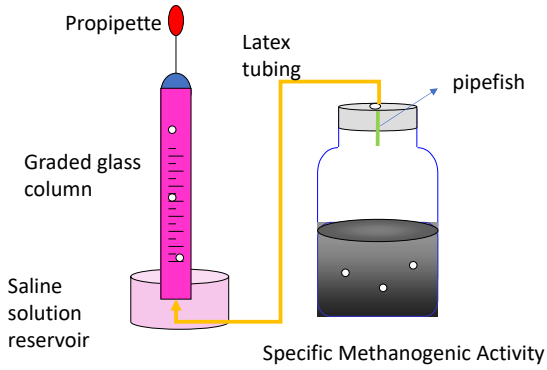

Figure 1. Measurement of the produced biogas.

The volume of the produced methane was calculated by multiplying the biogas volume by the proportion of methane in the biogas.

Composition of the biogas was determined by taking a sample from the headpiece of the bottle and injecting it into a gas chromatograph (GOW-MAC Instrument Co., Bethlehem, PA, USA) with an SP-4290 integrator and a stainless-steel column packed with carbosphere. The operating conditions of the chromatograph were: injector temperature at 443.15 K, the column at 413.14 K, the detector at 463.15 K, flow of the carrier gas (He) 500×10^{-9} m^3 $\cdot$ s^{-1}, filaments' current of 120 mA.

2.3 Determination of the inhibitor's concentration to reduce 50% de SMA (IC_{50})

Assays were performed keeping the same operating parameters described in the previous section on SMA, additionally, different amounts of disinfectants were added, as shown in Table 1. The IC_{50} is defined as the disinfectant concentration needed to diminish 50% the methanogenic activity of the inoculum.

2.4 Determination of the kinetic parameters

The velocity of methane production (R) was estimated from the initial slope of the curve of the accumulated production of methane using the minimal squares method. The confidence interval of this slope was calculated with a probability of 0.95. Then, the methanogenic activity (SMA) was determined with equation 1.

Table 1. Inhibition assay for the three disinfectants.

Disinfectant	Concentration ($kg \cdot m^{-3}$)
Benzalkonium	0.30×10^{-3}
	0.78×10^{-3}
	1.60×10^{-3}
Anibac	38.00×10^{-3}
	77.00×10^{-3}
	154.00×10^{-3}
Bactium	1.10×10^{-3}
	2.80×10^{-3}
	5.60×10^{-3}
	8.40×10^{-3}

$$SMA = \frac{R}{CF \cdot V \cdot VSS} \quad (1)$$

Where: SMA is specific methanogenic activity (kg $COD_{CH4} \cdot kg^{-1}VSS \cdot d^{-1}$), R is the velocity of methane production (m^3 $CH_{4accumulated} \cdot d^{-1}$), CF is the conversion factor (0.418 m^{-3} $CH_4 \cdot kg^{-1}$ COD, considering wet methane at 308.15 °C, Field, 1986), V is the volume of the liquid in the bottle (m^3), VSS is the concentration of the inoculum ($kg\ VSS \cdot m^{-3}$).

The SMA curves as a function of the acetate concentrations were obtained from the experimental results and the kinetic parameters were estimated, SMA_{max} and K_S, of equation 2, by means of the minimal squares of the reciprocals of SMA and acetate concentration (Ac).

$$SMA = \frac{(SAM_{\max} \cdot Ac}{K_S + Ac} \quad (2)$$

3 Results

3.1 *Acclimatation of the inoculum*

The initial SMA of the inoculum was of 0.44 kg $COD_{CH4} \cdot kg^{-1}$ VSS $\cdot d^{-1}$. After seven successive feedings, SMA reached 0.99 kg COD_{CH4} kg^{-1} VSS $\cdot d^{-1}$; experiments were performed at this SMA. The SMA increments during the successive feedings are shown in Figure 2.

The IC_{50} obtained for each disinfectant is shown in Table 2. To determine the inhibition constant, K_i, of each disinfectant, the determined IC_{50} value was used as dose of the inhibitor.

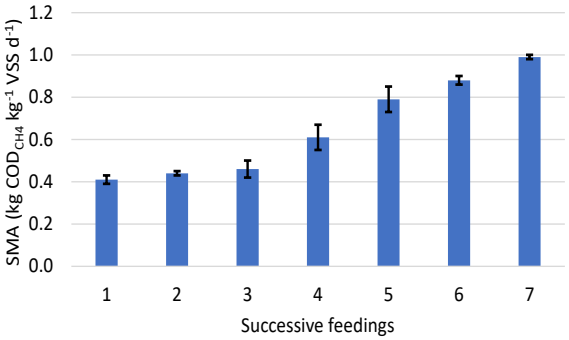

Figure 2. Variation of the specific methanogenic activity (SMA) during activation of the inoculum. The black lines represent the standard deviation of 24 independent test.

Table 2. Percent inhibition of the assayed disinfectants

Disinfectant	Concentration ($kg \cdot m^{-3}$)	Inhibition (%)
Benzalkonium	0.30×10^{-3}	12
	0.78×10^{-3}	16
	1.60×10^{-3}	50
Anibac	38.00×10^{-3}	7
	77.00×10^{-3}	14
	154.00×10^{-3}	58
Bactium	1.10×10^{-3}	3
	2.80×10^{-3}	9
	5.60×10^{-3}	19
	8.40×10^{-3}	55

3.2 *Estimation of the kinetic parameters of the inoculum*

Figure 3 shows the methane production at different initial concentrations of COD. The value of methane production velocity (R) was estimated by means of the initial slope of accumulated methane against time. Once R was determined, the SMA was calculated and plotted against the initial COD concentration (Figure 4).

With the average SMA, the kinetic parameters, SMA_{max} and K_S, of Eq. 2 were estimated by means of the minimal squares of the reciprocals of SMA and acetate concentration, Figure 5.

Knowing the slope and the intercept, the values of SMA_{max} and K_S can be determined as follows:

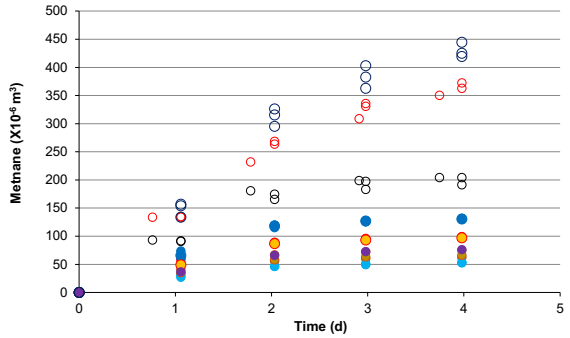

Figure 3. Production of methane from anaerobic sludge fed with different initial concentrations of sodium acetate trihydrate expressed as kg COD · m^{-3} (10.5 o, 8.9 o, 4.9 o, 2.9 ●, 2.3 ●, 1.6 ●, 1.4 ●, 1.1 ●).

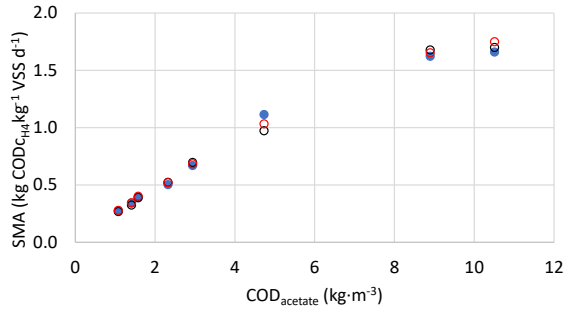

Figure 4. Specific methanogenic activity (SMA) at different initial concentrations of sodium acetate trihydrate in RAMM; three replicates per concentration.

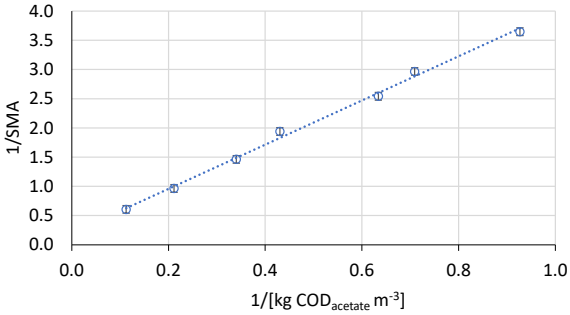

Figure 5. Graph of the average of the reciprocals of SMA and acetate concentration of the data of Fig. 4. Slope, m = 3.7761 and the ordinate at its origin, b = 0.2042, with r^2 = 0.994.

$$\frac{1}{SMA_{\max}} = b \qquad \frac{K_S}{SMA_{\max}} = m$$

$$SMA_{\max} = \frac{1}{b} = \frac{1}{0.2042} = 4.90 \qquad K_S = m \cdot MEA_{\max} = 3.7761 \cdot 4.90 = 18.49$$

Then: SMA_{max} = 4.9 kg COD_{CH4} ·kg^{-1} VSS · d^{-1} and K_S = 18.49 kg·m^{-3}.

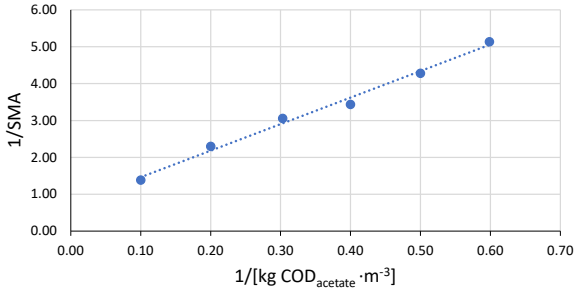

Figure 6. Graph of the reciprocals of SMA and acetate concentration, using 1.56×10^{-3} kg · m^{-3} of benzalkonium chloride as inhibitor. The slope, m = 7.1951, the ordinate at the origin, b = 0.7438, with r^2 = 0.991.

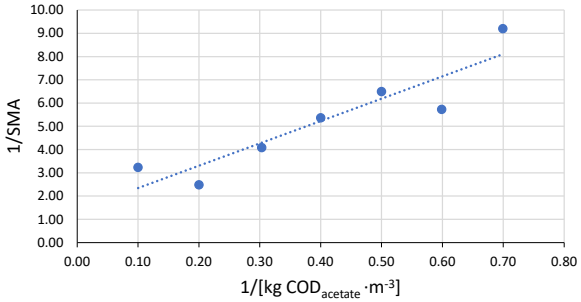

Figure 7. Graph of the averages of the reciprocals of SMA and acetate concentration using 8.40×10^{-3} kg · m^{-3} of bactium as inhibitor. The slope, m = 9.5975 and the ordinated at the origin, b = 1.3866, with r^2 = 0.842.

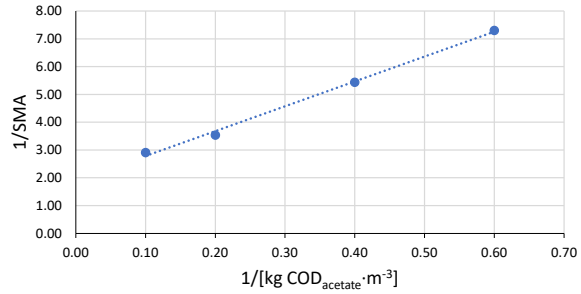

Figure 8. Graph of the averages of the reciprocals of SMA and acetate concentration, using 154×10^{-3} kg · m^{-3} of anibac as inhibitor. The slope, m = 8.9377, the ordinate at the origin, b = 1.8929, with r^2 = 0.9968.

3.3 Estimation of the inhibition constants

Any substance that reduces the velocity of a biochemical reaction can be considered an inhibitor. Inhibition studies allow knowing how the kinetic parameters of a biochemical reaction change and provide information on the kinetic mechanism of the reaction. Figures 6, 7, and 8 show the graphs of the reciprocals of MEA plotted against the reciprocal of

Table 3. Kinetic parameters for the methane production kinetics from acetate with different inhibitors.

Inhibitor	$SMA_{\max}$ (kg $COD_{CH4} \cdot kg^{-1}$ VSS $\cdot d^{-1}$)	k_S (kg $\cdot m^{-3}$)
without inhibitor	4.9	18.49
Benzalkonium chloride	1.34	9.67
Bactium	0.721	6.92
Anibac	0.53	4.72

Table 4. Values of K_i and α for the three inhibitors.

Inhibitor	K_i (kg·m^{-3})	α
Benzalkonium chloride	1.72×10^{-3}	0.34
Bactium	5.44×10^{-3}	0.27
Anibac	113×10^{-3}	0.10

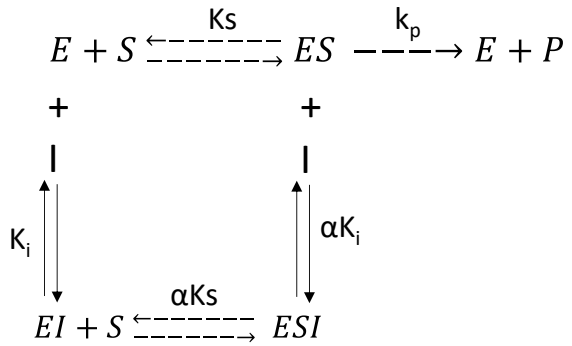

Figure 9. The simplest schematic representation for the mixed-type inhibition.

the acetate concentration assayed with benzalkonium chloride, bactium, and anibac, respectively. With the slopes and ordinates at the origin for each disinfectant, the values of SMA_{max} and K_S were determined, Table 3.

As observed in Table 3, the values of both SMA_{max} and K_S change in the presence of the inhibitor; hence, the inhibition could by uncompetitive or mixed, because both the Ks and SMA_{max} diminish. The decrement is not proportional, that is, the relation SMA_{maxapp}/SMA_{max} differs from the relation K_{Sapp}/K_S for the three inhibitors tested, 0.27, 0.52 for benzalkonium; 0.15, 0.37 for bactium, and 0.11, 0.26 for anibac. Because in uncompetitive inhibition, the diminution of K_S and SMA_{max} should be proportional, uncompetitive inhibition is discarded. Hence, data indicate that the inhibition exerted by the disinfectants is mixed.

In the mixed-type inhibition, the presence of the inhibitor (I) in the enzyme changes the dissociation constant of S from K_S to αK_S. Figure 9 shows a schematic representation of the mixed-type inhibition.

Where K_S, αK_S, K_i, and αK_i are the dissociation constants, k_P is the constant for the velocity of product formation.

$$K_S = \frac{[E][S]}{[ES]}, \alpha K_S = \frac{[EI][S]}{[ESI]}, \; K_i = \frac{[E][I]}{[EI]}, \quad \alpha K_i = \frac{[ES][I]}{[ESI]} \tag{3}$$

Then:

$$\frac{SMA}{SMA_{\max}} = \frac{[S]}{K_S(1+[I]/K_i)+[S](1+[I]/(\alpha K_i))} \tag{4}$$

Hence, the reciprocal of this equation allows obtaining K_i from the value of the slope at a given concentration of I:

$$m = \frac{K_S}{SMA_{\max}}\left(1+\frac{I}{K_i}\right) \tag{5}$$

And α is obtained from the ordinate at the origin:

$$b = \frac{1+\frac{I}{\alpha K_i}}{SMA_{\max}} \tag{6}$$

Values of K_i and α obtained for the inhibitors are shown on Table 4.

Conclusions

The IC_{50} for the tested disinfectants were 1.60×10^{-3}, 154×10^{-3}, and 8.4×10^{-3} kg $\cdot$ m^{-3} for benzalkonium, anibac, and bactium, respectively.

The values of the kinetic values determined for the tested disinfectants indicate that the type of inhibition exerted on the used microbial consortium is of the

mixed type, which would implicate that for any concentration of the inhibitor, a high concentration of the biodegradable substrate cannot improve the performance of the consortium.

Nomenclature

IC_{50}	Disinfectant concentration needed to diminish 50% the methanogenic activity of the inoculum [kg·m^{-3}]
SMA S	pecific methanogenic activity [kg COD_{CH4} · kg^{-1}VSS · d^{-1}]
R	Velocity of methane production [m^3 $CH_{4accumulated}$ · d^{-1}]
CF	Conversion factor [0.418 m^3 CH_4 · kg^{-1} COD, considering wet methane at 308.15 °C]
V	Volume of the liquid in the bottle [m^3]
VSS	Concentration of the inoculum [kg VSS · m^{-3}]
SMA_{max}	Maximum SMA [kg COD_{CH4} · kg^{-1}VSS · d^{-1}]
K_S	Half saturation constant [kg·m^{-3}]
Ac	Concentration of acetate [kg COD·m^{-3}]
K_i	Inhibition constant [kg·m^{-3}]
I	Concentration of the inhibitor [kg·m^{-3}]
k_P	Constant for the velocity of product formation
M	slope of the equation of the line
b	Intercept on the *y* axis

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