Growth Kinetics and Toxicity of *Enterobacter cloacae* Grown on Linear Alkylbenzene Sulfonate as Sole Carbon Source

**Khaled M. Khleifat · Khaled A. Tarawneh ·
Mohammad Ali Wedyan · Amjad A. Al-Tarawneh ·
Khalid Al Sharafa**

Abstract A successful attempt was made to isolate linear alkylbenzene sulfonate (LAS)–degrading bacteria from soil irrigated with wastewater. The isolated bacteria were able to use LAS as sole carbon and energy source. Maximum growth rates on LAS reached only 0.27 h^{-1}. 16S-rRNA sequencing and fatty-acid analysis placed the bacteria in the genus *Enterobacter cloacae*. The growth curves of *E. cloacae* both in the presence of and the absence of LAS were monitored using measurements of optical density at 600 nm in two different media, nutrient broth and M9 minimal medium, and were modeled mathematically. Growth in NB fit the Riccati and Voltera models, indicating that LAS is not toxic to *E. cloacae* cells. However, growth of *E. cloacae* in LAS-containing MM fit the Riccati and Voltera models, whereas growth in LAS-free MM fit the Riccati model only. Furthermore, the kinetic data shown were modeled by Monod's, Andrew's, and Tessier's specific growth rate equations, coupled with the rate of consumption of different concentrations of LAS as sole carbon and energy source, and we determined that Andrew's model best fit these data adequately as a result of the cell-inhibitory effect.

Introduction

The pollution of soil and water with xenobiotics is widespread in the environment and is creating major health problems. The use of microorganisms to biodegrade organic compounds from a polluted environment represents a potential solution to such environmental problems [1]. Linear alkylbenzene sulfonate (LAS) is a widely applied anionic surfactant that accounts for approximately 25% to 30% of the world's total synthetic surfactants. It is used in domestic as well as industrial detergent formulations and thus is discharged into wastewater collection systems worldwide [2, 3]. Commercial LAS is a mixture consisting of approximately 20 different compounds of closely related homologues and isomers, each containing an aromatic ring, sulphonated at the para-position, attached to a linear alkyl chain [4, 5].

Biodegradation is the most important mechanism for the complete removal of chemicals from the environment. It is nature's way of getting rid of wastes by breaking down organic matter into nutrients that can be used by other organisms [6–8]. Insufficient biodegradation leads to the development of great masses of foam in streams and rivers [3, 7–9]. Therefore, all efforts must be directed to biodegrade high concentrations of LAS [3]. Many studies have been reported, but until now, no studies have confirmed the degradation of high concentrations of LAS [10]. Most research has been performed using a mixed-culture consortium.

Biodegradation of a chemical in soil is determined by its bioavailability, the presence and size of its degradation populations, and the environmental conditions that affect its activity [11]. Primary biodegradation by individual cultures and culture consortium has been established by measuring the disappearance of LAS in the culture [10, 12]. These studies showed that ≥ 2 cultures in a consortium complement each other as long as their combined degradation ability is much greater than that accomplished alone by individual cells, indicating catabolic cooperation

K. M. Khleifat (✉) · K. A. Tarawneh · A. A. Al-Tarawneh ·
K. Al Sharafa
Department of Biology, Mutah University, Mutah, Karak, Jordan
e-mail: alkh_kha@hotmail.com

M. Ali Wedyan
Department of Biology, Al Hussein Bin Talal University, Maan,
Jordan

between the consortium members [10]. We report the isolation and characterization of bacterial strain *E. cloacae* using standard methods as well as 16S-rRNA and fatty-acid analysis. This strain was capable of growing on LAS as sole carbon and energy source. A mathematic model was used to interpret the growth of this isolate on different concentrations of LAS.

Materials and Methods

Bacterial Strain Isolation

Soil samples were obtained from Al-Kherba Al-Samra, which is located near Amman, Jordan. A total of 100 g moist soil was mixed in sterile distilled water (1:1, w/v) for 1 hour at room temperature. One milliliter of that suspension was cultured into 50 ml nutrient broth (NB) or M9 minimal medium (MM) [14]. Both media were initially supplied with 50 ppm LAS and adjusted to pH 7. The growth of bacteria was checked by observing optical density at 600 nm (OD_{600}) for 72 ± 12 hours. One-milliliter aliquots were aseptically inoculated into 50 ml MM supplemented with increasing concentrations of LAS to a maximum of 300 ppm. Growth was also checked by determining colony forming units per milliliter; this was done using dilution plate counts on nutrient agar medium. After growth had been achieved in 300 ppm LAS, selection was continued by consecutive inoculation into 50 ml MM with LAS as sole carbon and energy source. The bacterial growth was measured by spectrophotometer at OD_{600}. The 1-ml samples of each enrichment were kept at $-70°C$. The growth conditions were 37°C, 150 rpm, and pH 7.4. After growth in 300 ppm LAS, the resulting population was plated onto solid MM (1.5% agar) containing 100 ppm LAS as sole carbon and energy source. The bacterial isolates that grew in 100 ppm LAS were purified; colony morphologic characteristics were noted; and biochemical identity was determined using the REMEL kit (RapID ONE and RapID NF plus systems; Remel, Lanexa, KS) procedure.

Amplification of 16S rRNA Genes

Polymerase chain reaction (PCR) was performed as previously described [15]. The 16S rRNA gene-sequencing experiments were kindly performed by the Meningitis and Special Pathogens Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA.

Each final PCR (100 μl) contained 5 U Expand DNA polymerase (Roche, Indianapolis, IN), 2 μl DNA template, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl2, 200 μl (each) dATP, dCTP, dGTP, and dTTP, and 0.4 μl

each eubacterial primers F8 (5′ AGTTTGATCCTGGCT-CAG 3′) and R1492 (5′ ACCTTGTTACGACTT3′) [16]. Reaction mixtures were first incubated for 5 minutes at 95°C. Then 35 cycles were performed as follows: 15 seconds at 94°C, 15 seconds at 50°C, and 1 minute 30 seconds at 72°C. Reaction mixtures were then incubated at 72°C for an additional 5 minutes. PCR products were purified with a Qiaquick PCR purification kit (Qiagen, Valencia, CA).

16S rRNA Gene Sequencing

Sequencing primers were chosen from a panel of eubacterial primers: F8, R1492 (described previously), F357 (5′ TACGGGAGGCAGCAG 3′), R357 (5′ CTGCTGCCTCC CGTA3′), F530 (5′ CAGCAGCCGCGGTAATAC 3′), R530 (5′ GTATTACCGCGGCTGCTG 3′), F790 (5′ ATT AGATACCCTGGTAG 3′), R790 (5′ CTACCAGGGTA TCTAAT 3′), F1068 (5′ GTCGTCAGCTCGTGTCGT-GAG 3′), F1083 (5′ CGTGACATGTTGGGTTAAGTC 3′), F981 (5′ CCCGCAACGAGCGAACCC 3′), and R981 (5′ GGGTTGCGCTCGTTGCGGG 3′) [21-23]. Four additional eubacterial primers were used: R1333 (5′ CTAGCG ATTCCGACTTCATGC 3′), R180 (5′ TCTCTCAAGA CGTATGCGGTA 3′), R591 (5′ CATCCTGCTTAAGTAA CCGTC 3′), and F1127 (5′ ATTAGTTGCCATCATTCA GTT 3′). Sequencing was performed with the Applied Biosystems (ABI, Foster City, CA) BigDye terminator cycle sequencing kit (version 2.0) according to the manufacturer's instructions, with the exception of using 0.25 μl BigDye instead of 8 μl. Sequencing products were purified using Centri-Sep spin columns (Princeton Separations, Adelphia, NJ), and the sequencing products were resolved with an ABI model 3100 automated DNA sequencing system (ABI).

Evaluation of the 16SrRNA Gene Sequences of *Enterobacter* spp by Computer Analysis

16S rRNA gene sequencing of the amplified products, which was obtained with PCR primers F8 and R1492, generated 929-bp–long sequences that had 98.5% identity to the sequence of previously published *Enterobacter* spp. 16S rRNA gene sequences and 98.1% identity match to various *Klebsiella pneumoniae* sequence entries. The software used for all data analysis was GCG Wisconsin, version 10.2 (Accelrys, San Diego, CA). The raw trace files from the ABI 3100 sequencer were visually examined, aligned, and edited (SEQMERGE). *E. cloacae* sequences were compared using DISTANCES to determine the level of similarity among them. The sequences made in this study were compared with previously published *Enterobacter* spp. and *Klebsiella pneumoniae* sequences by using BESTFIT to determine levels of identity between sequences.

Fatty-acid Analysis

Fatty-acid analysis experiments were kindly conducted by T. L. Pitt at the Laboratory of HealthCare Associated Infection, Microbiology Division London, UK. Fatty-acid analysis (MIDI-Sherlock) of cell extracts results placed the organism in the genus *Enterobacter* with the highest match to *E. cloacae*. Therefore, the bacterial strain isolated was considered to be *E. cloacae*

Growth Conditions

In all experiments conducted, LAS-containing uninoculated media served as control. NB and agar medium were used for isolation of bacteria and in some degradation tests performed. MM was used to verify the degradability of LAS by the bacteria used in the study. MM as described by Miller [14], consisted of the following: 3.0 g Na_2HPO_4, 1.5 g KH_2PO_4, 1.0 g NH_4Cl, and 0.5 g NaCl dissolved in 500 mL distilled water with pH adjusted to 7.4 with 6 N NaOH. Then the following compounds were added: 0.24 g $MgSO_4$, 0.05 g $CaCl_2 \cdot 6H_2O$, and 0.05 g $FeCl_3 \cdot 6H_2O$. After autoclaving, the following were added by predissolving each in 2 mL distilled water and sterilizing by filtration through a 0.45-μm Millipore filter: 0.001 g thiamine-HCl. LAS (300 ppm) was used as carbon and energy source. The initial cell mass (starting inocula) for each experiment for the NB and MM media experiments were grown from single colonies in two consecutive shake flask cultures (overnight followed by 3-hour recovery) in NB. Cells from 3-hour cultures were harvested by centrifugation and then washed with and resuspended in NB at a concentration of 0.5 OD_{600} units. One milliliter of these bacterial cells was used to inoculate 50 ml of either media in 125-ml Erlenmeyer flasks for shake-flask experiments. Samples were then taken at time intervals and assayed for cell mass at optical density at OD_{600}. Growth curves using OD_{600} and viable counts for specific growth rates calculations were performed as previously described [3, 13].

Analytic Methods

LAS was analyzed in culture media using the classic methylene blue method [17], modified by Li and Rosen [18], as previously described [3]. The disappearance of LAS was qualified by reverse-phase high-performance liquid chromatography (HPLC) on a Spherisorb ODS2 column (250 × 4.6 mm; 5-μm particle size; motile phase 0.11 M $NaClO_4$ with acetonitrile gradient [0% to 100% vol/vol]), and compounds were detected spectrophotometrically at 255 nm [3].

Results and Discussion

Bacterial Identification

Using 16S rRNA gene sequencing, a 929-bp sequence was recovered. BLAST analysis indicated a 98.5% identity match to various *Enterobacter* spp. sequences and a 98.1% identity match to various *K. pneumonia* sequence entries. However, fatty-acid analysis placed the strain in the genus *Enterobacter* with the highest match to *E. cloacae*. Based on these procedures, along with the API system results, we concurred that the bacterial strain belongs to *E. cloacae*. To our knowledge, ours is the first study of LAS biodegradation by *E. cloacae*. In fact, although microbial degradation of LAS compounds has been discussed widely in the literature, no studies involving *E. cloacae* have been published.

Effect of Substrate Concentration on LAS Biodegradation by *E. cloacae*

Different substrate concentrations were used to investigate their effect on the biodegradation level of LAS (Fig. 1a). It was apparent that ≥300 ppm LAS resulted in <60% biodegradation. As a result, an inverse relation between the extent of LAS concentration and extent of biodegradation was shown. Decreased LAS degradation at higher concentrations was unlinked to a toxicity effect on the bacterial culture as predicted from the Andrews model in Eq. 7 and as reported with white rot fungus *Phanerochaete chrysosporium* [18]. To determine the exact time point for achieving complete degradation of 100 ppm LAS (Fig. 1b), LAS biodegradation was assessed at 2-hour time point intervals. It was found that complete degradation of 100 ppm LAS was achieved within 18 hours (Fig. 2b) compared with the results of a previous study showing degradation as occurring within days [8]. The 300-ppm LAS concentration resulted in the highest rate of LAS degradation (6.25 ppm/h).

Growth Curves and Mathematic Interpretation

E. cloacae cells were grown in NB and in NB plus 300 ppm LAS (Fig. 3a and b). In the second culture (MM), LAS was used as sole carbon and energy source (Fig. 4 and Table 2). Cells grown in LAS-containing NB had a shorter lag phase than that observed in cells grown in LAS-free NB media. Any growth of *E. cloacae* cells in LAS-containing MM was a function of exhaustion of LAS substrate because no other carbon source existed in MM, thus confirming LAS biodegradation as established by HPLC results (Fig. 2). Any further possibility for elimination of this anionic surfactant from the culture by surface adsorption or extensive chemical degradation is doubtful [3]. Moreover,
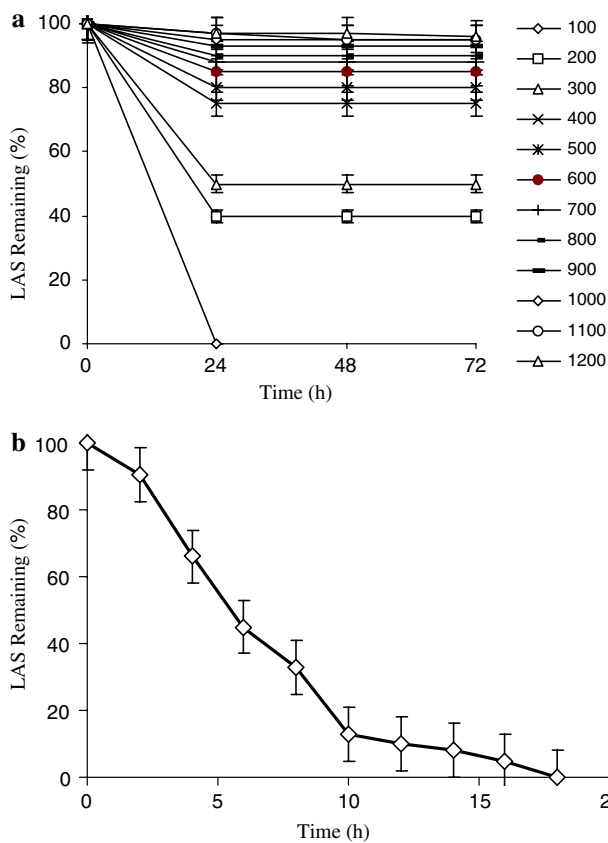
Fig. 1 Effect of different substrate concentration on the biodegradation of LAS by *E. cloacae*. (**a**) data collected at 24 h intervals, (**b**) data collected every 2 h intervals. The figure represents the percentage of remaining LAS expressed as active matter under the same experimental conditions (37°C, pH 7.4 and agitation rate of 150 rpm). All data are average of three trials with error bars indicating STDEVs (σ_{n-1})

the lag phase for cells grown in MM was shorter than the same counterpart cells grown in NB. It has been reported that single bacterial isolates are known to have limited ability for degrading the alkyl chain, and they also lack the capability to cleave the sulfonated aromatic ring that exists in the LAS aromatic ring [3, 8].

The inhibitory influence of substrate has been described by two types of growth kinetics, the Riccati and Voltera models (Fig. 3a and b). This growth was modeled based on increasing cell mass with time in the presence and the absence of population history (OD_{600} measurements). The Riccati model assumes that the inhibition of the cells is directly proportional to the square of population growth according to Eq. 1:

$$dx/dt = k\left(x - \beta x^2\right) x(0) = xo,$$ (1)

where β is a constant related to the percentage of inhibition, and x is the cell mass. This Riccati equation can be integrated to give the logistic curve according to Eq. 2:

$$x = \frac{x_o e^{kt}}{1 - \beta x_o(1 - e^{kt})}.$$ (2)

The logistic curve predicted in Eq. 2 shows a sigmoidal profile, which leads to a stationary population of size $x_s = 1/\beta$. In contrast, the Volterra model is used to predict a phase of decrease after the stationary population has exhausted all viable resources. It can be described thusly in Eq. 3:

$$\frac{dx}{dt} = kx(1 - \beta x) + K_o \left| \int_0^t x(r)dt \right| x(0) = 0,$$ (3)

where K_o is a constant account for the history or memory of population. The sign, K_o, is taken as negative for compounds that inhibit growth and positive for compounds that promote growth. The cell populations in LAS-free NB (Fig. 3a) and in LAS-containing NB (Fig. 3b) were the same, indicating that LAS had less toxicity on these cells. Both the Riccati and Voltera models clearly fit the experimental data adequately. The corresponding model parameters are listed in Table 1. Similar results were obtained when comparing these results with cells grown in MM. However, the Riccati model showed the best fit to the experimental data compared with the Voltera model, especially for the MM control (i.e., LAS-free MM) (Table 2). Experimental data for the growth of cells in MM plus 300 ppm LAS showed an adequate fit for both models. It is obvious from these experimental data that cells have little memory effect on growth, indicating that they can resist the toxicity of the LAS. Kramer et al. [20] found that *E. cloacae* tolerate up to 25% sodium dodecyl sulfate rather than metabolize it.

Toxicity of LAS Toward Bacterial Cells

A series of batch experiments were conducted based on viable cell number (Fig. 5) to determine toxicity of *E. cloacae* toward LAS. In these experiments, initial LAS concentrations varied from 500 to 3,000 ppm. Taking into account the effect of these concentrations on cell number, the specific growth rate, μ, can be determined experimentally by determining the ratio of the difference in cell concentration to difference in time [21] according to Eq. 4:

$$\mu = \frac{\ln(X_2/X_1)}{t_2 - t_1}.$$ (4)

The Monod, Tessier, and Andrews models describe the correlation between substrate concentration and specific growth rate as long as saturation kinetics for cellular systems can be formed. The Monod model has the following form (Eq. 5):

Fig. 2 HPLC chromatograms
showing the finger print of: (**a**)
standard solution of LAS
(100 ppm), (**b**) complete
degradation of LAS (100 ppm)
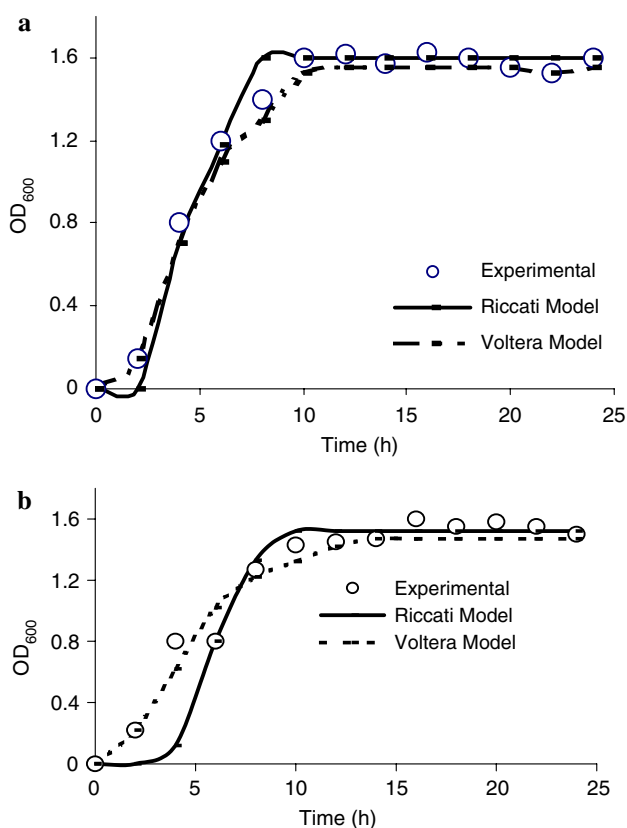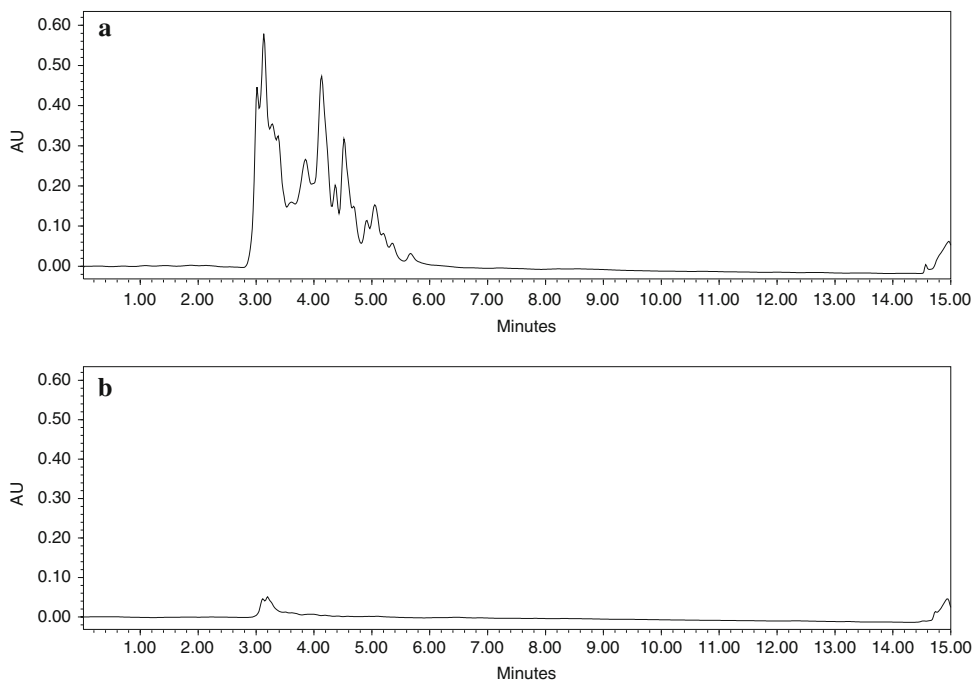
Fig. 3 Growth curve of *E. cloacae* on (**a**) nutrient broth media (LAS-free NB) and (**b**) LAS-containing NB medium. The curve represents the absorbance at 600 nm as a function of time. Data were fitted by *Riccati* and *Volterra* models; this was done under the same experimental conditions (37°C, pH 7.4 and agitation rate of 150 rpm)
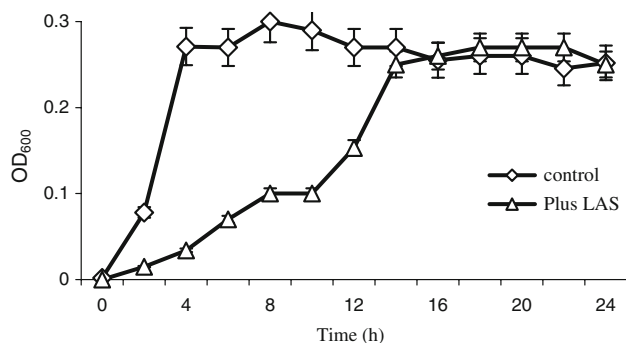

Fig. 4 Growth curve for *E. cloacae* grown on LAS-free minimal medium (M9) and 300-ppm LAS-containing M9 minimal medium. Growth was conducted under 37°C, pH 7.4 and agitation rate of 150 rpm

Table 1 Ricatti and Voltera model parameters for cells grown in NB and NB plus 300 ppm LAS

Parameters\model name	k	β	K_0
Riccati	1.6	0.67	–
Voltera	0.58	0.75	0.027

Table 2 Ricatti and Voltera model parameters for cells grown in MM and MM plus 300 ppm LAS

Parameters\model name	k	β	K_0
Riccati	3.4	4.2	–
Voltera	0.91	3.6	0.0001
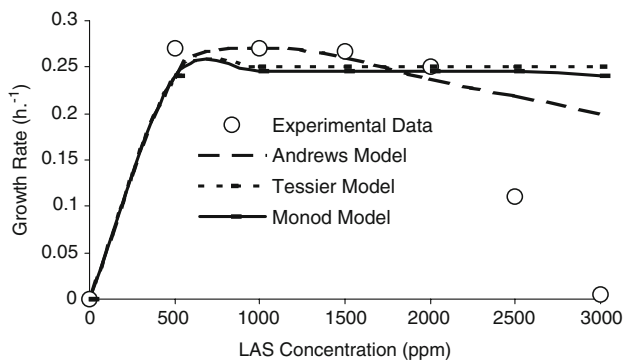
Fig. 5 The relationship between substrate concentration and growth rate, this carried out by three different models, all the models could fit the experimental data (*Monod*, *Tessier* and *Andrews*) being *Andrews* the best fit, this was done under the same experimental conditions (37°C, pH 7.4 and agitation rate of 150 rpm)

$$\mu = \frac{\mu_{max} S}{K_s + S}, \quad (5)$$

where μ_{max} is the maximum specific growth rate when $S \gg K_s$. More related forms of specific growth rate dependence have been proposed, which, in particular instances, give better fits to experimental data. For example, Tessier and Andrews, respectively, suggest the following models (Eqs. 6 and 7):

$$\mu = \mu_{max}(1 - e^{-S/K_s}). \quad (6)$$

The specific growth rate may be inhibited by medium constituents, such as substrate or product. This inhibition effect may be predicted by Eq. 7:

$$\mu = \frac{\mu_{max} S}{K_s + S + S^2/K_I}. \quad (7)$$

The specific growth curve that describes the relation between substrate concentration and growth rate for cells is shown in Fig. 5. The inhibition constant for the substrate (Table 3) is relatively high (1600 mg/l) which is a measure of sensitivity to inhibition by LAS indicate that the inhibition by LAS is negligible. Moreover, all of the models could fit these data. The corresponding parameters are listed in Table 3. Decreased growth rate may have been caused by the production of several inhibitory metabolic intermediates or by decreasing bioavailability of LAS to

bacterial cells with increasing LAS concentration. This bacterial isolate is thus interesting, and distinct model systems for the study of LAS biodegradation and kinetics could be useful. Finally, *E. cloacae* may be helpful in the bioremediation of LAS-contaminated soil and water.

Table 3 Model parameters that fit the experimental data showing the relation between substrate concentration and specific growth rate

Parameter	Monod model	Tessier model	Andrews model
μ_{max}	0.25	0.26	0.27
α	–	–	2.35
K_s	0.1	0.3	600
K_i	–	–	1600

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