Biodegradation of linear alkylbenzene sulfonate by a two-member facultative anaerobic bacterial consortium

Khaled M. Khleifat *

Mutah University, Department of Biology, Karak, Mutah 61710, Jordan

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Abstract

A bacterial consortium capable of degrading the linear alkylbenzene sulfonate (LAS) was isolated from the wastewater treatment plant. The bacterial consortium consisted of two members, Pantoea agglomerans and Serratia odorifera 2. Cells were grown evenly together in a minimal medium (M9) and nutrient broth (NB). The bacterial consortium was able to grow in the minimal medium containing LAS as the only carbon source. The percentage degradation of 200 ppm LAS by this bacterial consortium was better when cells were grown in NB (~70%) than in the M9 medium (36%). Also, the degradation ability by the bacterial consortium was very much higher than by its individual cells. This work shows that the two bacteria complement each other in the degrading ability of LAS, indicating catabolic cooperation between the two consortium members. An incubation temperature of 32°C, an agitation rate of 250 rev min⁻¹, and the addition of different carbon and nitrogen sources all independently caused complete mineralization of 200 mg L⁻¹ LAS within 48–72 h.

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1. Introduction

Linear alkylbenzene sulfonates (LAS) are widely used in surfactant formulation. The biodegradability of these compounds has been intensively studied, particularly in marine and wastewater treatment plants [1–4]. Most of the studies deal with primary biodegradation. In this initial step of the biodegradation, short-chain sulfophenyl carboxylate (SPC) residue appears as transient intermediates after the first terminal oxidation of the alkyl chain, which is then shortened by means of β-oxidation, causing the loss of surfactive characteristics [2]. SPC’s are then completely biodegraded in the second mineralization step, achieved by other organisms through desulfonation and a ring-opening process [5–7]. Much work has been done to confirm the complete biodegradability of LAS [3,8], however, little is known about the degradation processes and the microbial community involved [4]. The hypothesized facile degradation of LAS is more complex than previously speculated. LAS is not a single compound, but, typically, a mixture of 20 isomer compounds, all subterminally substituted, a linear alkyl chain (C₁₀–C₁₃) carrying a 4-sulfophenyl moiety.

Most LAS is discarded as sewage and efficiently removed (up to 99%) during sewage treatment [9], in some areas raw sewage that contains LAS is discharged directly into the environment [10]. Thus the consequence of the insufficient biodegradation of LAS is the development of huge masses of foam in streams and rivers in the vicinity of dams and other obstructions [8,11]. The aim of this work was to study the ability of two bacteria (Pantoea agglomerans and Serratia odorifera 2) to facilitate the biodegradability of LAS by individual isolates and their bacterial consortium under different conditions. The two kinds of bacteria had already been isolated from the wastewater treatment plant located on our University campus [12]. The conditions include pH, temperature, aeration and carbon and nitrogen sources. This is therefore the first report describing these two species as being able to degrade LAS. P. agglomerans has been previously described by many researchers [13,14]. It is ubiquitous in nature, animals and humans. Pantoea showed its best results in being able to use kerosene, toluene and vaseline as the carbon sources for growth. It is gram-negative, noncapsulated, nonsporeforming and motile rode. S. odorifera 2 has been
described by Grimont et al. [15]. It is a member of the normal flora of the gram-negative, small rod-shaped bacteria of the order Eubacteriales. These motile peritrichous bacteria are common inhabitants in water, soil, manure, bedding and feed. This group can be distinguished from other genera that belong to the family Enterobacteriaceae by its production of three special enzymes: DNase, lipase and gelatinase.

2. Materials and methods

2.1. Materials

LAS (linear alkylbenzene sulfonate) was supplied by Jordan Sulfochemicals Co. Ltd. Jordan. It was an aqueous sodium salt solution with a minimum purity of 96.5% and an average molecular weight of 320 g mol⁻¹ that was used in the preparation of working standards. Benzethonium chloride (benzamidine) and patent blue (disulfide blue) were supplied by Acros Organics, (Fisher Scientific, UK). Most of the chemicals used were either from Sigma, USA or from Fluka Chemika, Switzerland. Other chemicals were of analytical grade and were obtained from commercial suppliers.

2.2. Bacterial isolates

Two previously studied wastewater isolates, P. agglomerans (formerly Enterobacter agglomerans) [13] and S. odorifera 2 were used in this study [12]. Their morphological characteristics were re-verified and their biochemical identity was verified using the RISU kit (Rapid™ GNI and Rapid™ NF plus systems) procedure.

2.3. Analytical methods

LAS (linear alkyl benzene sulfonate) was analyzed in culture media using the classic methylene blue method [17], modified by Li and Rosen [18]. The LAS was extracted in an organic solvent (chloroform) and titrated against standardized benzamidine (benzamethionin) in the presence of patent (anionic dye). The endpoint was detected by the pink to blue oily droplets colour development of the organic layer.

The disappearance of LAS was qualified by reverse-phase HPLC on a Spherisorb ODS2 column (250 by 4.6 mm, particle size, 5 μm); the mobile phase was 0.11 M NaClO₄ with an acetonitrile gradient (0–100%; vol./vol.), and compounds were detected spectrophotometrically at 255 nm [11].

To test the effect of agitation rates on the LAS degradation by the bacterial consortium, the effect of different substrate concentrations (200, 300, 400, 500, 600 and 700 ppm) on the LAS removal by the bacterial consortium (P–S) was conducted. The growth medium (NB plus corresponding LAS concentration) was incubated at 37 °C incubation temperature, under a 150 rpm shaking rate and a pH of 7.5. Different pHs (5.5, 6.5, 7.5 and 8.5) of the growth media were used to assess the effect of variations in pH on the percentage degradation of LAS by bacterial consortium (P–S). The effect of different incubation temperatures (25, 32, 37 and 42 °C) upon the percentage degradation of LAS by bacterial consortium (P–S) was investigated.

In all experiments conducted, the LAS-containing uninoculated media were taken as the control. The nutrient broth (NB) and agar media were used for the isolation of bacteria and all degradation tests performed. The nutrient agar and nutrient broth were supplied by Difco. The ingredients of NB (g L⁻¹) are 5 g peptone, 5 g sodium chloride, 1.5 g beef extract and 1.5 g yeast extract. In the initial experiments, the LAS compound was used as the sole carbon and energy sources by including it in the M9 minimal medium (MM), which has no other organic compounds. This medium was used to further verify the degradability of LAS by the bacterial consortium. The minimal broth medium, as described by Miller [18], consists of the following: 3.0 g NaHPO₄, 1 g KH₂PO₄, 0.5 g NH₄Cl, and 0.5 g NaCl were dissolved in 500 mL distilled water and the pH adjusted to 7.4 with 6N NaOH. Then the following compounds were added: 0.24 g MgSO₄, 0.05 g CaCl₂, 6H₂O, and 0.05 g FeCl₃·6H₂O. After autoclaving, the following were added by predissolving each in 2 mL deionized water, and then sterile-filtering: 0.05% thiamine–HCl, 0.01% MnSO₄, 0.01% ZnSO₄·7H₂O, 0.01% FeCl₃·6H₂O, and 0.1% MgSO₄·7H₂O. After autoclaving, the following were added by predissolving each in 2 mL deionized water, and then sterile-filtering: 0.05% thiamine–HCl, 0.01% MnSO₄, 0.01% ZnSO₄·7H₂O, 0.01% FeCl₃·6H₂O, and 0.1% MgSO₄·7H₂O.
Fig. 1. Biodegradation of LAS by *Serratia odorifera*, *Pantoea agglomerans* and their mixed culture (P–S) grown on nutrient broth (NB) and minimal medium (MM). The culture was incubated at 37 °C, 150 rpm shaking rate and pH of 7.5. The control consisted of uninoculated broth plus 0.2 g L$^{-1}$ LAS. The data are the average of four independent experiments.

Fig. 2. Growth curve of *P. agglomerans* and *S. odorifera* and their consortium (P–S) on (a) nutrient broth; (b) minimal media. The inoculum was 0.1 mL of stationary phase grown into 50 mL nutrient broth. For the P–S culture they consisted of 50% each. Error bars in (a) and (b) indicate standard deviations; where not visible they are smaller than the diameters of the points.

Fig. 3. Effect of different substrate concentration on the biodegradation of LAS by mixed culture of *P. agglomerans* and *S. odorifera* as a function of time. The culture was incubated at 37 °C under 150 rpm shaking rate and pH of 7.5. The control consisted of uninoculated broth plus 0.2 g L$^{-1}$ LAS. The data are the average of three repeated experiments.

rial consortium (P–S) grown in NB was 70%, compared with that 36% in MM. In the case of the individual bacterial cultures, in NB and MM, the percentage LAS degradation ranged between 25 and 30% and 12 and 20%, respectively. These data indicate that the single bacterial culture contributes enzymatic activity for cleavage of the benzene ring and that of the two bacterial components of the mixture must be present to obtain significant degradation ability of the ring [1,5,10]. Moreover, these results were supported by using the minimal medium in which the combined culture were grown, and which can use LAS as carbon and energy sources (Fig. 1). Thus, the formation of any cell mass is a function of the exhaustion of this compound.

### 3.1. Growth curves

The growth curves of the *P. agglomerans*, *S. odorifera* and their bacterial consortium (P–S) that were grown in both LAS-containing nutrient broth and the minimal medium (Fig. 2a and b) reinforce the biodegradation ability data obtained in MM and nutrient broth, as illustrated in Fig. 1. The growth of three cases in the nutrient broth reached the stationary phase after 15 h (Fig. 2a), whereas in MM the stationary phase was achieved after 20 h and the exponential growth was preceded by acclimation periods of 9 and 6 h for individual cultures and their consortium, respectively (Fig. 2b). The growth of individual bacteria in the LAS-minimal media containing only LAS as the carbon source was poorer, based on the OD$\text{max}$ measurement, compared with the P–S co-culture. The growth of the P–S consortium reinforces the notion of higher degradation efficiency, compared with the growth of the individual bacterial cultures alone. Our results agree with those of Jiménez et al. [10], with the exception being that their bacterial consortium consisted of four gram-negative bacteria.

Our study thus represents the first report on LAS removal by this bacterial consortium (P–S). A huge body of research has suggested that optimization of the growth conditions could enhance the biodegradation of xenobiotics [19,20].

### 3.2. Effect of substrate concentration

Fig. 3 shows that the LAS concentration of 200 ppm resulted in the highest degradation percentage (68%). This percentage
Fig. 4. Effect of culture medium pH on the biodegradation of LAS by mixed culture of \textit{P. agglomerans} and \textit{S. odorifera} as a function of time. The culture was incubated at 37°C and under 150 rpm shaking rate. The control consisted of uninoculated broth plus 0.2 g L$^{-1}$ LAS. The data are the average of three repeated experiments.

of LAS degradation was completed within 96 h. However, the higher LAS concentration utilized resulted in the early termination of degradation activity. This concluded at almost the 48 h time point and at a lower percentage of degradation activity. The reason for the early termination of degradation with the LAS concentration above 200 ppm could be the result of an increase in membrane permeability that causes the dissipation of ion gradients and membrane potential or leakage of essential cell constituents[21]. Therefore the 200 ppm LAS concentration was taken as a fixed concentration in all experiments conducted.

3.3. Effect of pH

For the combined culture, the greatest LAS biodegradation removal was observed in the nutrient broth of pH 8.5 (90%) (Fig. 4) with about a 20% decrease at pHs 7.5 and 6.5. When the pH was 5.5, a 50% decrease in LAS biodegradation was obtained. The data thus show that a pH of 8.5 would be the optimum level for maximum LAS degradation by these combined bacteria. Pérez et al. [22] reported that, under aerobic degradation conditions, the LAS influences the pH self-regulation capacity, particularly when the LAS concentration is above 20 mg L$^{-1}$ and thus external neutralization is always required. This is true as long as the cell biomass for pH 7 without LAS had a higher growth biomass than that of pHs 8.5 or 5.5–6.5 (data not shown). The drop in pH was attributed to the production of acidic intermediates as a result of the LAS degradation[5]. This last observation provides further capacity for belief in the notion that biodegradation rather than sorption was primarily responsible for the LAS removal [21].

3.4. Effect of incubation temperature

The data on the percentages of degradation brought about by the co-culture under study (P–S) at different incubation temperatures are shown in Fig. 5. It is seen from the results that there was a rise in the percentage degradation of LAS, along with a rise in the temperature between 25 and 32°C, followed by an early steep fall (within 48 h) with a further rise in temperature from 37 to 42°C. The increase in temperature above the threshold point (37°C) may increase the microbial membrane toxicity as well as the growth itself being affected. The data therefore showed that a temperature of 32°C would be optimum for complete degradation of LAS, as confirmed by HPLC (data not shown). Generally speaking, temperature affects the rate of biodegradation of xenobiotics by influencing the physical and chemical properties of the LAS compound, microbial metabolism, the specific growth rate of microorganisms, the rate of enzymatic activity involved in the oxidation process and the composition of microbial community[23,24].

3.5. Effect of agitation rate on the biodegradation of LAS

At high aeration (250 rev min$^{-1}$), the combined culture caused complete degradation within 72 h of incubation time (Fig. 6). However, at normal aeration, only 70% of the...
degradation was achieved within a 96 h interval, whereas when cells were grown under limited aeration (75 rev min\(^{-1}\)) less than 40% of LAS degradation was obtained.

It should be considered that degradation is basically an aerobic process and hence the introduction of air to the solution will favour it. Aerobic degradation of LAS is preferred due to the fact that the first stage of degradation, oxygenation at the end of the alkyl chain, requires oxygen [5,8,10]. Other studies indicate that LAS can be anaerobically degradable only if preceded by a period of aerobic exposure [26].

### 3.6 Effect of carbon and nitrogen source

All of the carbon sources being tested caused an increase in the LAS biodegradation rate (Fig. 7). Sucrose, maltose and glucose were the better carbon sources, although all carbon sources being tested produced better and more complete degradation activity than the positive control (without C-sources). This complete degradation was achieved within 72 h.

All nitrogen sources being tested produced the greatest rate of LAS degradation, which was completed within 48 h (Fig. 7). The nature of the carbon and nitrogen sources may affect both the growth biomass and enzymes involved in LAS degradation. The fact that the first stage of degradation, oxygenation at the end of the alkyl chain, requires oxygen [5,8,10]. Other studies indicate that LAS can be anaerobically degradable only if preceded by a period of aerobic exposure [26].

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[6] S. odorifera 2 as a function of time. The culture was incubated at 37 °C, under 150rpm shaking rate and pH of 7.5. The control consisted of unamended broth plus 0.2 g/L LAS. The data are the average of three repeated experiments.


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