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Isolation and Identification of Anionic Surfactant Degrading Bacteria from Activated Sludge

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ABSTRACT

Background: Linear alkylbenzene sulfonate (LABS) is an anionic surfactant widely used all over the world. They will eventually end-up and accumulate in household or industrial sewage. Due to their high foaming capabilities which can cause numerous problems in sewage treatment facilities as well as direct toxic effects on many different organisms in ecosystem; they are generally considered as serious pollutants. Many reports have indicated that common bacteria can readily degrade LABS. **Methods:** In this survey, two different bacteria were isolated from Tehran municipal active sludge that showed the ability to degrade LABS rapidly and actively upon using it as their sole source of carbon. Biochemical tests as well as 16S rRNA gene sequencing performed. **Results:** Results have indicated the two isolates to be *Acinetobacter johnsoni* and *Pseudomonas beteli*. After experiments to optimize the pH and temperature for growth of the two bacterial isolates, the extent of LABS, utilization was evaluated by HPLC method. The *Pseudomonas beteli* and *Acinetobacter johnsoni* isolates were able to degrade 96.4% and 97.2% of the original LABS levels after 10 days of growth, respectively. Mixed culture of the two isolates did not significantly increase LABS utilization (97.6%). **Conclusion:** Our study showed the ability of two isolated steains to rapidly biodegrade LABS under aerobic conditions. *Iran. Biomed. J. 11 (2): 81-86, 2007*

Keywords: Linear alkylbenzene sulfonate (LABS), Biodegradation, Active sludge, Anionic surfactant

INTRODUCTION

Anionic surfactants such as linear alkyl benzene sulfonates (LABS) which have higher sudding and biodegrading potentials are more often used recently [1]. These compounds can complicate and cause problems in sewage aeration and treatment facilities due to their high foaming and lower oxygenation potentials [2]. The molecular structure of LABS is composed of three units: (1) a hydrocarbon chain (C_{11}-C_{14}); (2) a benzene ring attached to the chain; and, (3) a sulfonated group attached to the ring [3]. The most important ecological property of any surfactant is the relative ease of their biodegradation [4]. Biodegradation is most often performed by soil or aquatic microorganisms and leads to generation of water and carbon dioxide gas [5]. LABS are β-oxidized by microorganisms to sulfophenyl carboxylate and a new surfactant called linear alkyldiphenyl ether disulfonate which in turn gets converted to sulfodiphenyl ether carboxylate (Fig. 1) [6]. A *Pseudomonas aeruginosa* strain (W51D) was isolated with the ability to mineralize linear alkylbenzene sulfonate (LAS) at a significant rate [7]. An enrichment culture was established in a bioflow chemostat. Biodegradation in the chemostat was monitored by measuring changes in the concentration of methylene blue active substances (MBAS) [8] and by performing batch mineralization assays with ^{14}C- ring-labeled LAS [9].

In this survey, LABS degrading bacteria were isolated and identified by 16S rRNA sequencing from activated sludge of several locations in Tehran (Iran). Their single as well as mixed culture surfactant degradation capability in aerobic growth was measured by HPLC method.

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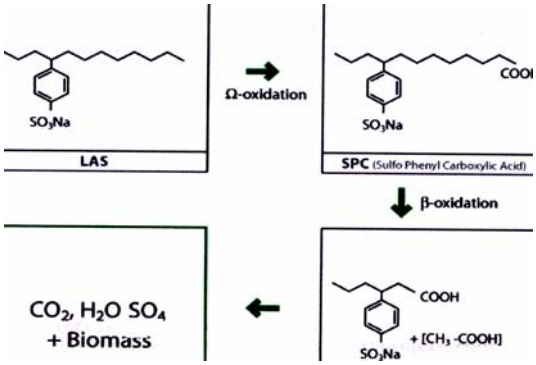

Fig. 1. Molecular structure of LABS and its metabolites.

MATERIALS AND METHODS

Bacterial isolation. Activated sludge samples were obtained from the secondary sedimentation tank in Gheitarihe Sewage Treatment Company (Tehran, Iran). The samples were subjected (5%) to 500 ml basal salt medium (BSM) (KH_2PO_4, 3.5 g; K_2HPO_4, 1.5g; NH_4Cl, 0.5 g; NaCl, 0.5 g; Na_2SO_4, 0.14 g and $MgCl_2.6H_2O$, 0.15 g dissolved in 1 liter of distilled water and the final pH adjusted to 7.1) and containing 1.5 mM commercial LABS (MW~ 288.38, CMC~2310 mg/l, Merck Co., Germany) as the sole carbon source. The inoculated media were incubated with constant shaking (150 rpm) at room temperature. After no foams were visible during growth (due to surfactants utilization), the liquid culture (OD_{650} = 1) was transferred to solidified (1% agar) BSM with 1.5 mM LABS in culture plates and incubated at 28°C. Following three subcultures on the solid media, two different bacterial colonies were isolated and identified. The growth curve for the two bacterial strains (MH1 and MH2) in the surfactants containing liquid media as well as pH and growth temperature optima were subsequently determined.

Culture. Each strain was grown, either as single or mixed culture, after an adaptation step in nutrient broth containing LABS, in BSM containing 1.5 mM LABS as the sole source of carbon. Incubation was performed at optimum pH and temperature with shaking (150 rpm) for 12 days. Culture samples were collected and analyzed for LABS utilization after 1, 3, 5, 7 and 10 days of growth.

Surfactant degradation. HPLC with a C-18 column (18 cm length and 4 mm width) using an isocratic mobile phase gradient of acetonitrile-water (80:20) was conducted at a flow rate 1 ml/min. Eluent absorption was detected with a UV spectrophotometer at 220 nm.

Bacterial identification. Initial identification schemes were performed with biochemical tests as suggested by the Bergeys Manual of Systematic Bacteriology. For final and specific identification, 16S rRNA sequencing was performed. For performing to molecular diagnostic technique, the following protocols were conducted. Bacterial cultures grown in LB medium at 35°C for 24h were used for DNA extraction. Following centrifugation of 1 ml of culture media (1500 ×g for 5 s). The bacterial pellet was suspended in 16 µl of cell lysis solution and incubated at 80°C for 5 minute. RNase A (3 µl) was then added and incubated at 37°C for 20 minute. The resulting suspension was subjected to phenol-chloroform extraction and following centrifugation (1500×g, 3 s); the purified DNA sample was collected. PCR with specific primers for 16S rRNA gene ($^{5'}$AGAGTTTGATCCTGGC$^{3'}$, $^{3'}$TACCTTGTTACGACTT$^{5'}$) was performed and followed by electrophoresis on agarose gel. DNA in the amplified bands was removed by GFX gel band purification kit (Genomic DNA Extraction Kit Cat.NO.YGT50S). The purified DNA sequencing from the electrophoresis gel was performed by Seq Lab Company (Germany).

RESULTS

The two bacteria (MH1 and MH2) that were isolated from activated sludge grow well in BSM media with LABS as their sole carbon source. The optimum pH values for the growth of MH1 and MH2 strains in the basic medium at 30˚C was 7.4 and 8.0, respectively. Based on morphologic and biochemical characteristics (Fig. 2 and Table 1), as well as 16S rRNA gene sequencing, with the nearest phylogenic relatedness, the MH1 and MH2 strains are members of *Pseudomonas betelli* and *Acinetobacter johnsoni* strains, respectively. Figure 2 shows electron micrographs (SEM) of the two bacteria in active growth. Figure 3 shows the electrophoresis gel of PCR amplification products.

HPLC analysis indicated that MH2 strain had the highest surfactant degrading potential (Figs. 4 and 5). The MH1 strain was able to decrease LABS level in the growth media from an original of 522 mg/l to

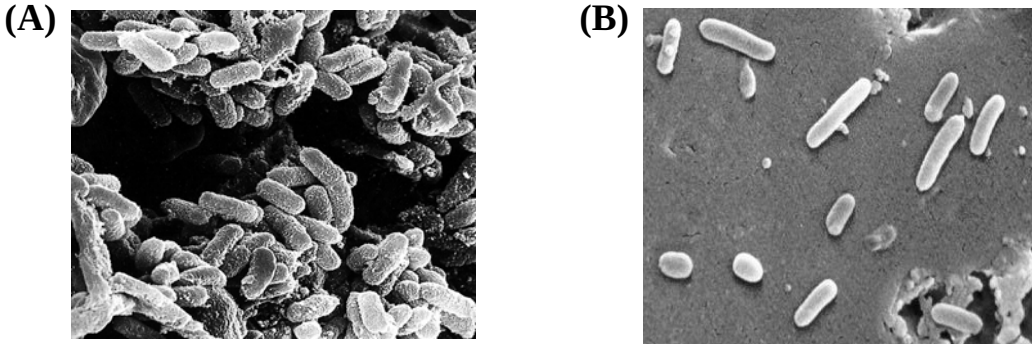

Fig. 2. Electron micrographs of MH1 strain **(A)** and MH2 strain **(B)**.

Table 1. Morphologic and biochemical characteristics of the isolated strains as suggested by the Bergeys Manual of Systematic Bacteriology.

Tests	MH1	MH2
Gram-staining	Slightly curved rods 0.5-1.0 µm in diameter, 1.5-5.0 µm in length, Gram -negative	Straight rods 0.9-1.6 µm in diameter, 1.5-2.5 µm in length, Gram-negative
Motility	+	+
Capsule	+	+
Oxidase	+	-
Catalase	+	+
Growth in:		
4˚C	+	-
42˚C	+	-
Haemolysis	+	-
Citrate	+	+
Acid from glucose	-	-
Nitrate reduction	+	+
Tryptophanase	-	-
Arginine dihydrolase	+	+
Gelatinase	+	-
Lysine hydrogenase	-	-
Pigment production	-	-
Utilization of:		
D-Lactose	-	+
Glutamate	+	-
Malonate	+	-
L-Ornithine	+	-
L-Leucine	-	-

the extent of 93.6% within 5 days; whereas, the other strain did so to the extent of 84.6% (Fig. 3). However, following 10 days of incubation, MH2 strain showed greater degradation (97.2%) potential relative to the MH1 strain (96.4%). The highest peak of LABS degradation occurred during the logarithmic phase of bacterial growth. Co-culture of the two strains did not significantlly increase the degradation potential (97.6% LABS degradation after 10 days of growth) relative to single bacterial growth (Table 2).

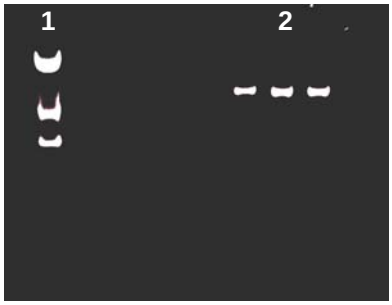

Fig. 3. PCR amplification of 16S DNA coding for rRNA of MH1 (lane 1) and MH2 (lane 2) pure culture on 1% agarose gels. The molecular weight marker was 2036 bp.

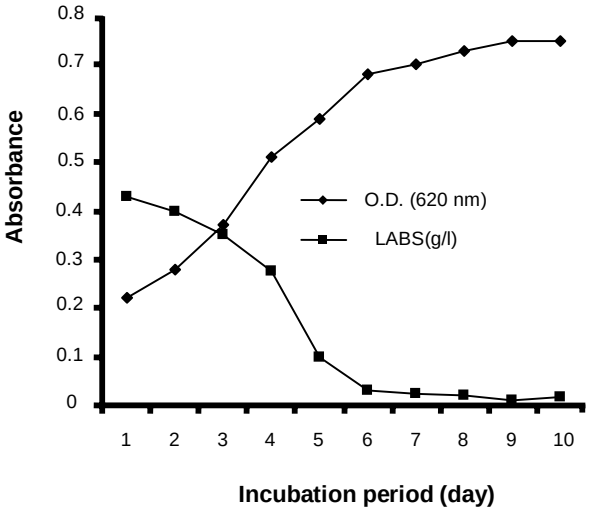

Fig. 4. Growth of MH1 strain in relation to LABS degradation.

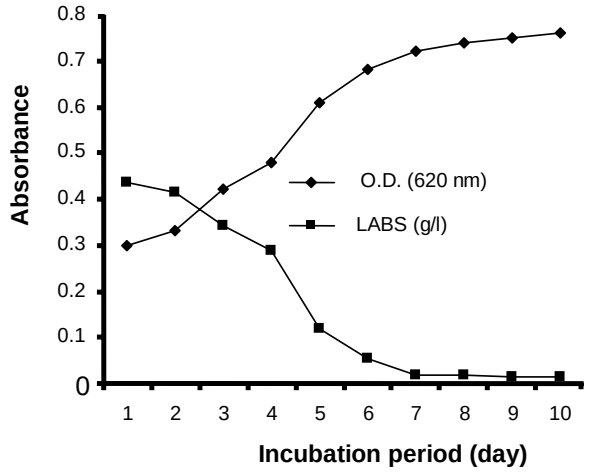

Fig. 5. Growth of MH2 strain in relation to LABS degradation.

DISCUSSION

Past experiences have demonstrated that anionic surfactants biodegradation are exclusively conducted by bacteria [10]. Investigators such as Schleheck, Jimenez, and Dhuib have used activated sludge cultures in order to isolate heterotrophic anionic surfactant degrading bacteria [3, 8, 11]. In this survey, aerobic cultures of activated sludge from Tehran municipality were performed in order to isolate anionic surfactant degrading bacteria. Activated sludge provides elevated nutritional carbon and other growth factors for growth of a wide variety of microorganisms; therefore it is an ideal source for isolation of specific bacteria capable of enzyme production or degradative potentials [4]. This is the main reason we sought to isolates surfactants degrading bacteria from activated sludge samples collected in Tehran municipality.

Two different bacteria were isolated after subsequent growth in BSA containing LABS as the sole carbon and energy source. Schleheck *et al.* [6] have used 16S rRNA gene sequencing for surfactant degrading bacteria identification. Whereas, Dhuib *et al*. [11] have relied solely on biochemical tests in order to identify their isolated bacteria. In this survey, we have used both biochemical as well as molecular methods in order to identify the two strains.

The maximum biodegradation capabilities of the two strains were displayed during logarithmic phase of growth. This is in agreement with the observation of Dhuib and Schleheck *et al*. [3, 11]. Hayashi *et al*. [12] have used a MBAS method for determination of anionic surfactant biodegradation in aquatic environment. This chromatographic method was originally proposed in 1976 and was subsequently used by many other investigators. [9, 11, 13].

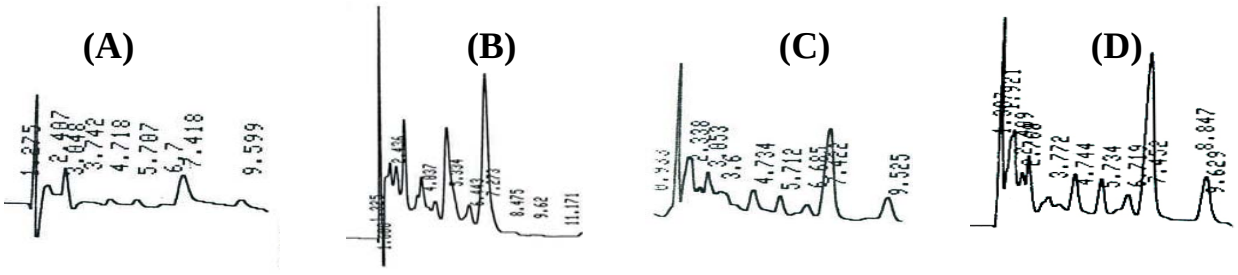

Fig. 6. HPLC analysis of LABS degradation by **(A)** MH1 strains at 1 days **(B)** MH1 strains at 10 days, **(C)** MH2 strains at 1 days, **(D)** MH2 strains at 10 days of incubation.

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Table 2. Results of HPLC analysis based on concentration and removal percentage of LABS from culture.

Incubation period (day)	Co-culture		MH2 isolate		MH1 isolate	
	LABS level (g/l)	Utilization LABS (%)	LABS level (g/l)	Utilization LABS (%)	LABS level (g/l)	Utilization LABS (%)
0	0.5	-	0.5	-	0.5	-
1	0.432	13.6	0.436	12.8	0.429	14.2
3	0.236	52.8	0.341	31.8	0.35	30
5	0.035	93.1	0.077	84.6	0.032	93.6
7	0.016	96.8	0.018	96.4	0.025	95
10	0.012	97.6	0.014	97.2	0.018	96.4

Jerebkova *et al*. have used this technique to evaluate anionic surfactant elimination by Pseudomonas biofilms [9]. In later years, Schleheck *et al.* [6] and Schulz *et al*. [14] have suggested that the presence of contaminating ions and intermediate compounds can inhibit precise detection of LABS levels by the methylene- blue assay. They suggested that HPLC is a superior technique for LABS identification.

Continued growth and biomass accumulation of the bacteria were coincidental in the media. This indicates that the bacteria are actually utilizing LABS as their sole carbon source. This is in agreement with the results of other investigators [5, 16, 17]. During stationary phase (7^{th}-10^{th} days of growth), no significant decrease in LABS levels was detected, indicating that bacterial growth had begun to level off. This was true for both MH1 and MH2 strains.

Jerebkova *et al*. [9] have noted that Pseudomonas cultures in continuous bioreactors have contributed to a 70% decrease in surfactant levels after 20 days. Other studies have noted different levels of surfactant utilization in closed cultures. For instance, over 90% of surfactant usage was noted by locally isolated Citrobacter spp. after 35 hours of growth [18]. In this survey, the MH1 strain was able to utilize 94% of the original LABS levels after 120 hours.

Sigoillot *et al*. [17] have reported that mixed cultures of different bacteria can dramatically improve the biodegradation potential. In this study, co-culture of the two isolated strains did not cause a dramatic rise in surfactant utilization. In conclusion, the results of this study suggest that growth of simple bacteria such as Acinetobacter or Pseudomonas in household and industrial sewage can be a cost-effective method of anionic surfactant elimination.

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