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Biodesulfurization of dibenzthiophene and petroleum by *Rhodococcus erythropolis*

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The removal of sulfur from petroleum by microorganisms has emerged as one of the most promising alternatives to hydrodesulfurization (HDS). The use of microorganisms offers the advantages of higher specificity towards organic sulfur, low cost and milder operating conditions. In this study, the biodesulfurization activity of seven bacterial strains was investigated using dibenzothiophene (DBT) as substrate. DBT is a compound, often found in petroleum, which resists hydrodesulfurization. Combinations of some of these bacteria were also examined for their biodesulfurization activity. Degradation products were detected using high performance liquid chromatography (HPLC) and mass spectrometry (MS) analyses. Of the seven strains tested, *Rhodococcus erythropolis* Q1a-22 exhibited the greatest biodesulfurization activity, converting DBT to 1-hydroxybiphenyl (1-HBP). The effect of two nonionic surfactants, Triton X-100 and Tween 80, was also investigated. Unlike Triton X-100, Tween 80 did not show toxicity towards *R. erythropolis* Q1a-22. Maximum degree of biodesulfurization was observed at the critical micelle concentration of this surfactant. *R. erythropolis* Q1a-22 was also found to desulfurize petroleum.

Keywords: acid rain; petroleum; biodesulfurization; dibenzothiophene; anionic detergent

INTRODUCTION

A primary cause of acid rain is the release of sulfur oxides to the atmosphere upon combustion of sulfur-containing fossil fuels. To protect the environment from the detrimental effect of these air pollutants, sulfur must be reduced, if not completely eradicated, from fossil fuels prior to combustion. Presently, the industrial approach involves a catalytic process known as hydrodesulfurization (HDS) to remove sulfur from crude-oil derived fuels. HDS converts organic sulfur to hydrogen sulfide gas by reacting crude oil fractions with hydrogen gas using inorganic catalysts such as Ni/Mo or Co/Mo supported on aluminum oxide, at pressures between 150 to 3,000 lb/in^2 and temperatures between 290°C to 455°C. Although HDS can remove various types of sulfur compounds (e.g., thiols, sulfides, and thiophenes), it is very costly, energy intensive and some of the heterocyclic organosulfur compounds (e.g., dibenzothiophene and benzothiophenes) are HDS resistant. These limitations of HDS have led to the development of alternative methods to this technology [5].

Biodesulfurization or the microbial removal of organic sulfur from fossil fuels, has been explored as an alternative to hydrodesulfurization due to its potential of being metabolically specific, ecologically benign and inexpensive [13]. Several bacterial isolates have been observed to remove sulfur from organic compounds, and these are of the genera *Brevibacterium, Gordona, Sulfolobus, Pseudomonas, Micrococcus, Cryptococcus, Rhodococcus, Thiobacillus,* and *Arthrobacter* [6].

Dibenzthiophene (DBT) is generally used as a substrate for biodesulfurization since it models a notably recalcitrant organic sulfur compound. Two primary pathways for DBT desulfurization have been proposed: the Kodoma pathway wherein the

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initial attack is directed against one of the carbon atoms and the 4S pathway, which was proposed by Kilbane, wherein the initial reaction is the oxidation of the sulfur atom, which is then followed by the cleavage of the C-S bond, the subsequent release of sulfate ion and the formation of the end product, 2-hydroxybiphenyl as shown in Fig. 1 [4]. In petroleum biodesulfurization, the microbial agent must solely utilize the sulfur and not the carbon sources in petroleum (4S pathway) to preserve the quality of the substrate.

Since DBT is a polycyclic aromatic compound, its availability in aqueous solution is hindered by its hydrophobic character. This low availability thus limits bacterial desulfurization of DBT. A possible way of enhancing the bioavailability of DBT is through surfactant application. Surfactants are substances known for their amphiphilic nature (apolar, hydrophobic 'tail', and a polar hydrophilic 'head' group). This amphiphilic character leads to the property of surfactant molecules to undergo self-association or micellization—a process wherein surfactant molecules arrange themselves into organized molecular assemblages known as micelles. Micelles are structures of surfactant molecules wherein the hydrophobic portion associate together to form the core from which solvent (H_2O) is excluded and the polar head groups are in contact with and hydrated by a number of water molecule [7]. Surfactants are known to increase the amount of hydrophobic compounds in the aqueous phase by incorporation into these surfactant micelles. Several studies have shown that nonionic surfactants increase the availability of polycyclic hydrocarbons in the aqueous phase [1]. However, only very limited investigations have been done on the effects of surfactants on biodesulfurization.

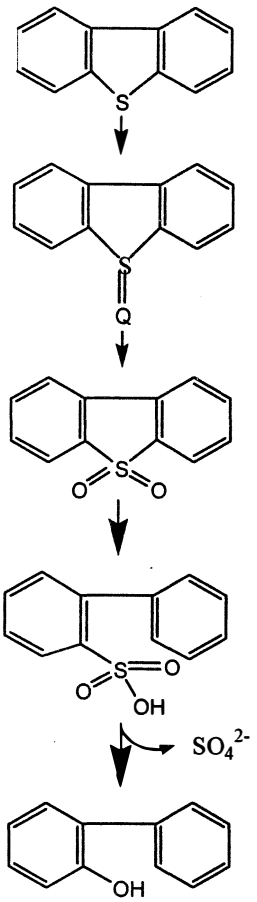

Fig. 1. 4S pathway of DBT biodesulfurization as proposed by Kilbane (1992).

In this paper, several bacterial strains were screened for biodesulfurization activity. The effect of a consortium of microorganisms on the rate of biodesulfurization of DBT was determined and the most effective strain on the biodesulfurization of petroleum was used. The effect of nonionic surfactants, Tween 80 and Triton X-100 on biodesulfurization were also studied.

EXPERIMENTAL

Screening of bacterial isolates. Seven bacterial isolates were screened for biodesulfurization studies: (1) *Arthrobacter luteus* BIOTECH 1077; (2) *Thiobacillus thiooxidans* BIOTECH 1850; (3) *Thiobacillus ferrooxidans* BIOTECH 1851; (4) *Pseudomonas putida* BIOTECH 1337; (5) *Rhodococcus erythropolis* Q1a-22; (6) *Rhodococcus erythropolis* N1-36; and, (7) *Rhodococcus erythropolis* N1-43. The first four bacterial cultures were obtained from the Philippine National Collection of Microorganisms (PNCM) in the National Institute for Molecular Biology and Biotechnology (BIOTECH), at the University of the Philippines, Los Baños, Laguna. All three cultures of *R. erythropolis*, isolates Q1a-22, N1-36 and N1-43 were obtained from Steven Krawiec of Lehigh University, Bethlehem, Pennsylvania and Ping Wang of Pennsylvania State University, University Park [12]. Selection of these bacterial species as test organisms for this present work was based on reported literature [8, 13].

The growth of these seven bacterial strains was monitored to determine whether the test organism is able to grow in the culture medium for desulfurization studies. The bacterial inoculum for desulfurization studies should be harvested at mid-log phase.

The following bacterial inocula were used for the growth studies: (a) 48-h culture of *A. luteus* grown in R. medium; (b) a 48-h culture of *P. putida*, grown in Nutrient Agar (Difco); (c) 4-day old cultures of *T. ferrooxidans* and *T. thiooxidans* cultured in 9K medium; and, (d) 4-day old cultures of the three isolates of *R. erythropolis* grown in L-B medium.

The culture medium used for the preliminary screening and growth studies is Mineral Salts Sulfur-Free medium (MSSF) [8]. This medium is composed of (per liter distilled water): 0.4 g of KH_2PO_4, 1.6 g of K_2HPO_4, 1.5 g NH_4Cl, 0.17 g of $MgCl_2 \times 6H_2O$, 0.09 g of $CaCl_2 \times 2H_2O$, 1 mL of vitamin solution and 5 mL of mineral solution. The vitamin solution contained (per liter distilled water) 100 mg of thiamine, 50 mg of p-aminobenzoic acid, 50 mg of vitamin B_{12} and 10 mg of biotin. 1 L of

mineral solution contained 1.5 g of nitrilotriacetic acid (dissolved in 500 mL water and adjusted to pH 6.5 with 10 M KOH), 5.1 g of $MgCl_2 \times 6H_2O$, 0.66 g of $MnCl_2 \times 2H_2O$, 1.0 g of NaCl, 1.0 g of $FeCl_3 \times 6H_2O$, 0.1 g of $CaCl_2 \times 6H_2O$, 0.01 g of $CuCl_2 \times 6H_2O$, 0.08 g of $ZnCl_2$, 0.05 g of $AlCl_3$, 0.01 g of H_3BO_3 and 0.04 g of Na_2MoO_4. The carbon sources used were 5 g/L of glucose and 5 g/L of sodium acetate. The sulfur source was 0.54 mM DBT.

For *T. ferrooxidans* and *T. thiooxidans*, 25 mL portion of the culture was aseptically pipetted out and transferred to flasks containing 250 mL MSSF medium with 0.54 mM DBT. For *A. luteus*, *P. putida*, and *R. erythropolis*, a loopful of the agar culture was transferred aseptically to flasks containing MSSF medium with DBT. Parallel set-ups were prepared without DBT. All inoculated flask cultures were done in triplicates. Uninoculated MSSF medium without DBT was used as negative control. Cultures and controls were shaken continuously and incubated at room temperature for 36 h. Organisms that showed growth indicated by turbidity in MSSF + DBT were selected for growth studies and subsequent desulfurization studies.

Growth monitoring experiment. Growth studies were performed to determine the mid-log phase of the selected test organisms, namely, *A. luteus* and the three *R. erythropolis* cultures, N1-36, N1-43, and Q1a-22. The bacterial inoculum for desulfurization should be harvested at mid-log phase.

For the growth studies, a 48-h culture of *A. luteus* in R medium and a 4-day old culture of *R. erythropolis* Q1a-22 grown in LB medium were used as inoculum. A loopful of each agar culture was inoculated onto flasks containing 100 ml MSSF + DBT (0.27 mM and 0.54 mM DBT) with the minimum glucose level required for the growth of the organism. Growth of the organism in MSSF without DBT was also monitored to compare the growth curve of cultures grown with and without DBT. All set-ups were prepared in triplicates. Uninoculated MSSF medium without DBT, prepared in duplicates, were used as negative control. Cultures and controls were incubated at room temperature for 48 h with shaking. Growth was monitored by taking 5 mL aliquot from the sample cultures and negative control solutions every hour and measuring its absorbance at $\lambda = 500$ nm using Perkin-Elmer double beam spectrophotometer. Absorbance readings correspond to the number of cells and are indicative of the growth of the organism. Absorbance values were plotted against the incubation period.

pH monitoring. Changes in the pH of cultures were measured using the same 5 mL sample. In previous studies, a decrease in pH was observed as a consequence of DBT biodesulfurization. The lowering of the pH is detrimental to the growth of microbial agents [12] hence, the pH of the culture should be monitored so that appropriate pH adjustments could be made and the growth and biodesulfurization activity of the organism would not be hindered.

Biodesulfurization of DBT by A. luteus and R. erythropolis. Biodesulfurization activity of four bacterial cultures which showed growth in MSSF with DBT, namely, *A. luteus* BIOTECH 1077 and *R. erythropolis* (N1-36, N!-43 and Q1a-22) was studied using shake-flask cultures. All bacterial test organisms were grown in 100 ml MSSF with 5 g sodium acetate, without DBT and shaken until mid-log phase. A 10% inoculum was used for this experiment. Thus, 25 ml of the bacterial culture was added to MSSF to make a 250 mL culture with 0.54 mM DBT. This was done in triplicates. Uninoculated MSSF medium without DBT in duplicates was used as negative control. Cultures were shaken continuously for 168 hours at room temperature.

DBT desulfurization by *A. luteus* and *R. erythropolis* were measured by HPLC using 5 mL aliquot from each replicate culture or control. To each aliquot, 20 mL acetonitrile was added. The resulting solutions were analyzed using HPLC operating under the following conditions: eluting solvent = 60% acetonitrile (HPLC grade), column = Hibar RP18, maximum pressure = 3000 psi, pump = A, flow rate = 1.0 mL/min, injection volume = 20 μL, λ = 254 nm, attenuation = 128, peak treshold = 1000, offset = 10 and chart speed = 0.1. The isolated components were then further analyzed using MS by direct injection.

Biodesulfurization of DBT using microbial consortia. Two microbial combinations were tested for DBT biodesulfurization namely: a) consortium 1 consisting of *R. erythropolis* Q1a-22 and N1-36 and b) consortium 2 consisting of *R. erythropolis* Q1a-22 and N1-43. Each test organism was grown in LB agar and incubated for 48 h at room temperature. This experiment was undertaken to determine if co-existence of the two microbial cultures would cause more efficient DBT desulfurization that is, greater DBT desulfurization in shorter period of time.

The microbial consortium was prepared by inoculating 100 ml MSSF with 0.54 mM DBT with a loopful of each of the two members of the cultures. Microbial consortia 1 and 2 were incubated for 30 h at room temperature with continuous shaking.

One mL aliquots of the 30-h microbial consortium were inoculated onto tubes containing 10 mL MSSF with 0.54 mM DBT. Triplicate inoculated tubes were set-up. Negative controls were prepared in duplicates. All tubes were incubated at room temperature for seven days with continuous shaking. After incubation, desulfurization of DBT was determined using HPLC and MS.

Desulfurization of DBT by R. erythropolis in the presence of nonionic surfactants. *R. erythropolis* Q1a-22 were grown until mid-log phase (30th hour) in MSSF medium (w/out nonionic surfactant). They were then harvested and 1 mL of this culture was inoculated into a fresh MSSF media to make a 10-mL solution. This media already contained a fixed amount of DBT as the sole sulfur source and nonionic surfactant in different concentrations for each set of samples. For the first set of

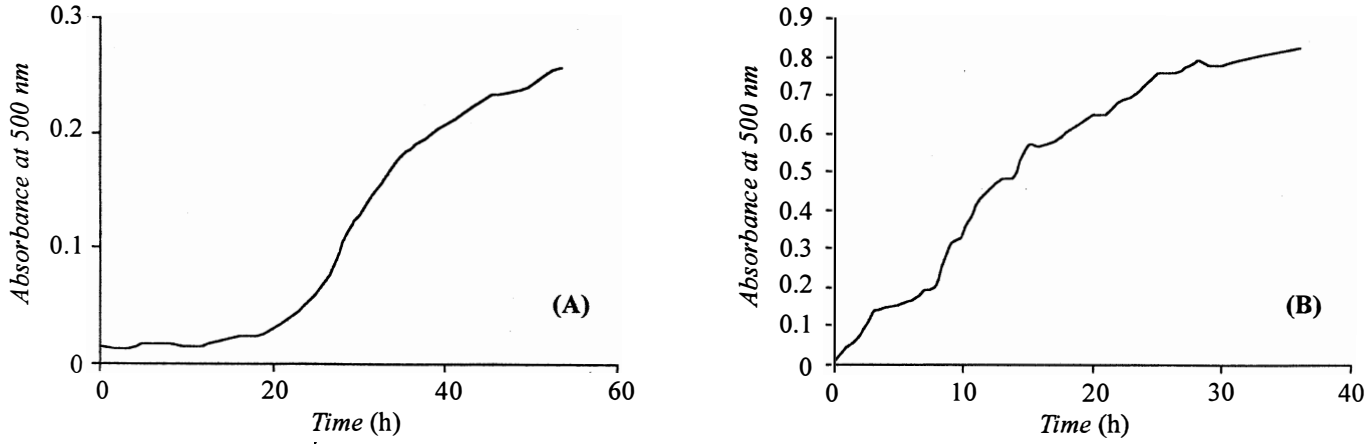

Fig. 2. Growth curves of (A) *A. luteus, and* (B) *R. erythropolis Q1a-22.*

samples Triton X-100, an alkylphenol ethoxylate, was added to the media then sterilized for 15 min to make sure no other bacteria will interfere the biodesulfurization of DBT by Rhodococcus. Similar procedure was followed for samples containing Tween 80, a polyoxyethelene sorbitan ester. Triton X-100: 0.0, 8.5×10^{-5}, 1.7×10^{-4}, 3.4×10^{-4}, 6.8×10^{-4} M (cmc = 1.7×10^{-4} M); Tween 80: 0.0, 6.25, 13, 25, 50, 100 mg/L (cmc = 13 mg/L).

Dibenzothiophene was prepared by dissolving the required amount of DBT in ethanol to make a 54 mM solution. 0.1 mL of this was then added to the sterilized MSSF. Biodesulfurization cultures were incubated for seven days (168 h) under room temperature with shaking at 200 rpm on a rotary shaker. For each set of analysis, three samples (with bacteria) and two controls (without bacteria) were prepared.

Calculations for DBT desulfurization. Desulfurization of DBT by bacterial strains was determined from the peaks in HPLC chromatograms of possible degradation products. These peaks were identified using mass spectrometry by direct injection method and quantified using the chromatograms of standard solutions.

Petroleum biodesulfurization by R. erythropolis. Desulfurization of petroleum by *R. erythropolis* Q1a-22 was investigated using shake flask cultures. The petroleum product was obtained from Caltex, Philippines. A loopful of a 30-h old LB agar culture of *R. erythropolis* Q1a-22 was inoculated onto flasks containing 100 mL MSSF with 5 mL gas oil. Triplicate cultures were prepared and controls were done in duplicates. Again, desulfurization was determined by evaluating the peaks in HPLC chromatograms of possible degradation products.

RESULTS AND DISCUSSIONS

Growth of test organisms in MSSF with DBT. Mineral salts sulfur-free medium (MSSF) was the medium selected in this study because it contains the essential mineral requirements of the test organisms. Moreover, the medium is sulfur-free, thereby inducing the organism to utilize the amended DBT or petroleum as source of sulfur. It was previously shown that DBT desulfurization by *R. erythropolis* N-36, N1-43 and Q1a-22, three of the seven isolates used in this present study, was not observed when a sulfur source (i.e., $MgSO_4$), apart from DBT, was added in the culture medium [14]. This proves that the enzymes involved in DBT desulfurization are likely inducible enzymes, which are produced only when there is no other available sulfur source.

Results of the growth studies showed that only *A. luteus* BIOTECH 1077 and *R. erythropolis* Q1a-22, N-36 and N1-43 were able to grow in MSSF with DBT. Growth was indicated by the increase in the turbidity of the medium. Turbidity is a clear indication of bacterial growth since bacterial cells act as colloidal suspension, which scatters light. Figure 2 presents the growth curves for *A. luteus* and *R. erythropolis* Q1a-22. The mid-log phase for *A. luteus* and *R. erythropolis* Q1a-22 occurred during the 8th and the 30th hour, respectively. Thus, an 8-h culture of *A. luteus* and a 30-h culture for *R. erythropolis* Q1a-22 were used as inocula for biodesulfurization studies.

No growth was observed for *T. thiooxidans* BIOTECH 1850, *T. ferrooxidans* BIOTECH 1851, and *P. putida* BIOTECH 1337. This observation indicates that the three organisms were unable to grow in MSSF with DBT as sole sulfur source. Thus, these organisms were not used in the subsequent biodesulfurization studies.

Changes in the pH of the medium were monitored since previous studies on desulfurization activities by microorganisms grown in DBT-containing medium reported a pH dependent biodesulfurization activity [13]. In this study, the pH of MSSF medium increased from pH 7 to pH 9 during the entire biodesulfurization process. This observation was most evident in the case of *R. erythropolis* Q1a-22. Desulfurization of DBT by *R. erythropolis* Q1a-22 occurred on day one when the pH changed from 7 to 9. Desulfurization increased significantly

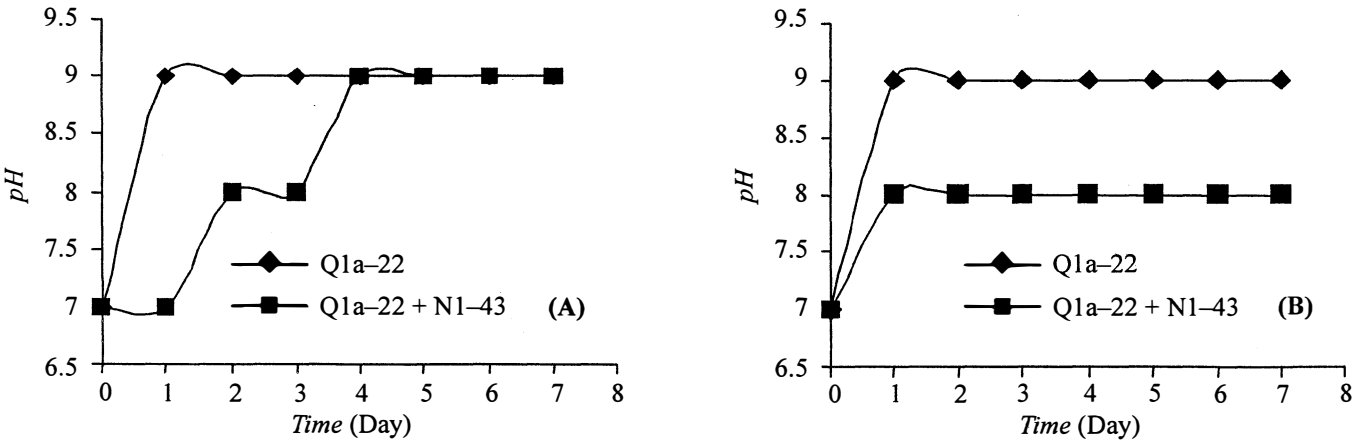

Fig. 3. The pH profile of biodesulfurization by R. erythropolis Q1a-22 at (A) *0.27 mM DBT, and* (B) *0.54 mM DBT.*

until day nine while the pH of MSSF remained at pH 9. A similar trend was also observed in the microbial consortia investigated (Fig. 3). However, previous studies reported a lower pH (acidic) of the medium as DBT degradation progressed [13]. But based on the mechanism that was proposed by Kilbane, biodesulfurization of DBT would yield sulfate ions (SO_4^{-2}), which could lead to a significant increase in pH as desulfurization progresses. Sulfate ions can hydrolyze in water to produce bisulfate (HSO_4^-) and hydroxide (OH^-) ions. The increase in pH observed in this study could be attributed to the formation of these OH^- ions.

Biodesulfurization activity of A. luteus and R. erythropolis. Biodesulfurization activity of *A. luteus* BIOTECH 1077 was first investigated using HPLC analysis at l = 254 nm. The chromatogram obtained revealed only one peak corresponding to DBT, based on the peaks obtained from DBT standards, indicating that *A. luteus* did not desulfurize DBT (data not shown).

HPLC was also used to determine desulfurization activity of *R. erythropolis* N1-36 and N1-43. The chromatogram obtained, just like that of A. *luteus,* only registered the peak which corresponds to DBT and not its degradation products. Therefore, we can infer that these two strains also failed to desulfurize DBT. The lack of desulfurization activity in both N1-36 and N1-43 was unexpected. N1-36 was extensively used by Wang and Krawiec [13] in understanding the kinetics of desulfurization. Cultures of N1-36, N1-43 and Q1a-22 were provided for this current study by these authors. Morphological examination of the cultures revealed their purity, thus contamination during transport could have not occurred. Identity of the cultures has been verified with the previous investigators.

R. erythropolis Q1a-22, however, showed significant desulfurization activity based on the HPLC chromatogram obtained. The isolate yielded three additional peaks with retention times of approximately 1, 2 and 6 min, corresponding to products of DBT biodesulfurization, aside from the peak found at 23 min (for DBT), as shown in Fig. 4. Fractions corresponding to the three peaks were isolated using 60% acetonitrile as eluting solvent. Desulfurization product corresponding to one peak with retention time of approximately 6 minutes, based on injected HBP standard, has been tentatively identified as o-hydroxybiphenyl (2-HBP).

The degradation of DBT and the presence of 1-HBP as the degradation product were confirmed by MS analysis. Peaks in the MS spectra are expected at 184 ± 1 m/z for DBT and 169 ± 1 m/z for 2-HBP. It is very evident, from the spectra obtained that DBT was degraded to 1-HBP since the peak at 184.9 m/z, which corresponds DBT, for the control was significantly reduced in the medium that contained *R. erythropolis* Q1a-22 and a new peak at 168.2 m/z, which corresponds to 1-HBP, was also observed (Fig. 5). Based on HPLC and MS analyses, we can conclude that *R. erythropolis* Q1a-22 follows the 4S

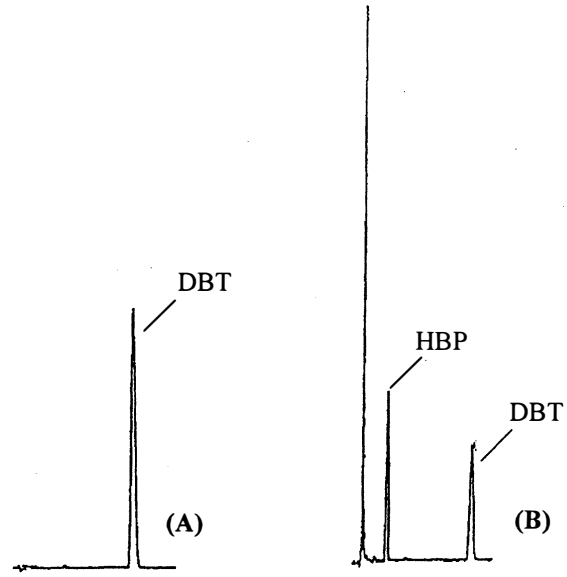

Fig. 4. HPLC chromatograms of DBT biodesulfurization (A) *without R. erythropolis Q1a-22 and* (B) *with R. erythropolis Q1a-22 at 0.54 mM DBT.*

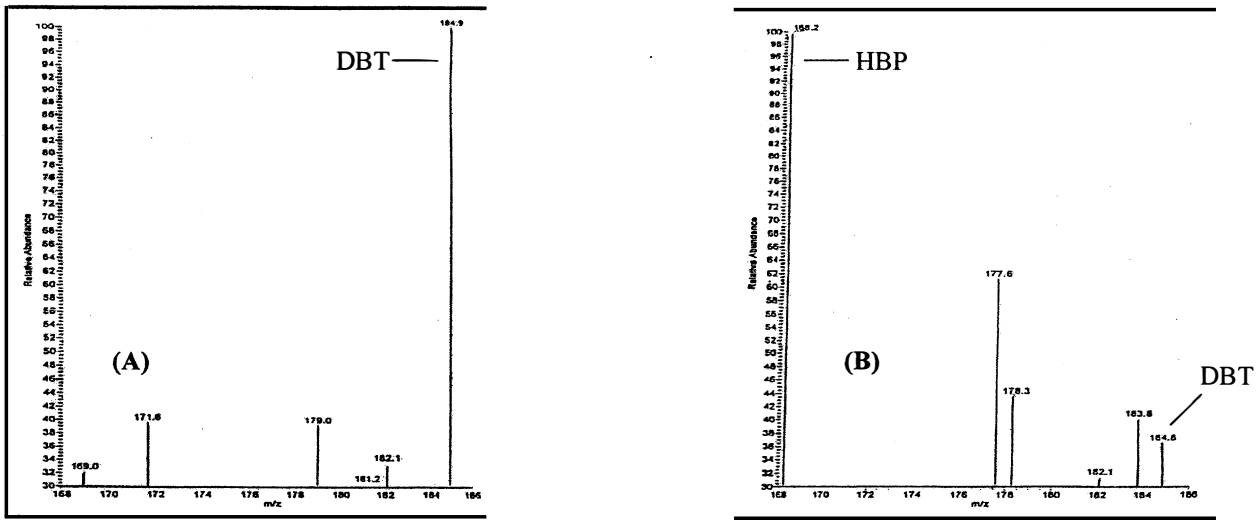

Fig. 5. MS spectra of DBT degradation (A) *without R. erythropolis Q1a-22 and* (B) *with R. erythropolis Q1a-22 at 0.54 mM DBT.*

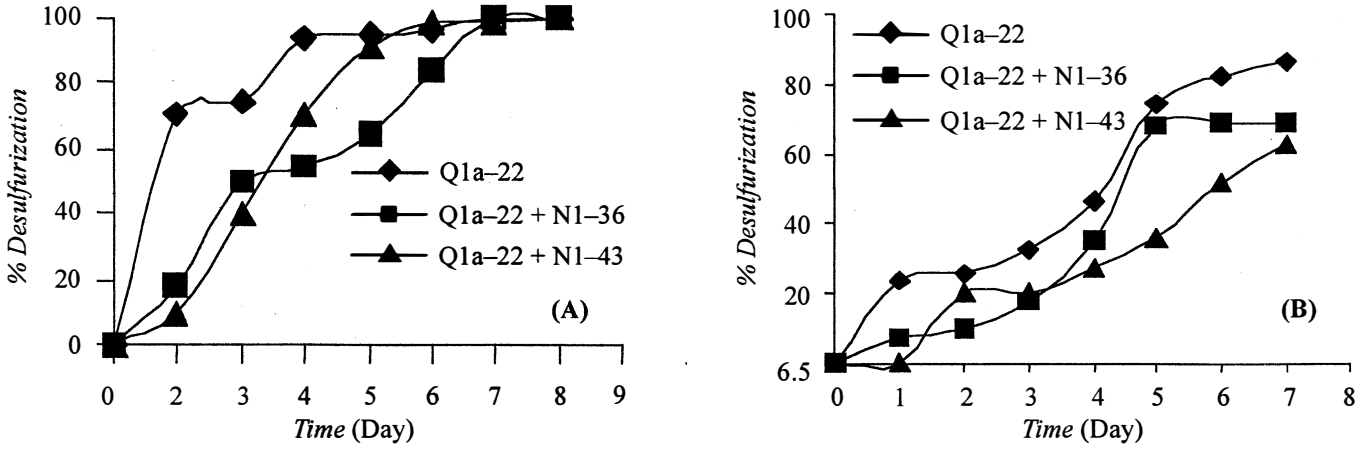

Fig. 6. Rate of DBT desulfurization at (A) *0.27 mM and* (B) *0.54 mM.*

DBT degradation pathway as proposed by Kilbane. *R. erythropolis* Q1a-22 degraded 100% and 87.24% of 0.27 mM and 0.54 mM DBT, respectively after seven days of culture (Fig. 6). These concentrations were based on a study to determine optimum concentration for growth.

Biodesulfurization activities of microbial consortia of Q1a-22 + N1-36 and Q1a-22 + N1-43 strains were assessed by HPLC. Chromatograms revealed about 30-36% decrease in DBT desulfurization. Consortium Q1a-22 + N1-36 desulfurized 69.55% of 0.54 mM DBT; consortium Q1a-22 + N1-43 yielded 83.49% DBT desulfurization (Fig. 6). This suggests that the two strains in the consortia decrease in DBT desulfurization could be due to competition of the two strains of *R. erythropolis* for nutrients.

Petroleum samples were treated with *R. erythropolis* Q1a-22. Unlike in the DBT biodesulfurization, the exact sulfur level in petroleum cannot be measured by HPLC since there are many forms of sulfur in the mixture. Still, the HPLC chromatogram obtained showed some distinct changes in the peaks after the petroleum was treated with *R. erythropolis* Q1a-22 indicating some bacterial activity as shown in Fig. 7. However, these metabolic products were not characterized further due to the complexity of the system.

The effect of two nonionic surfactants, Triton X-100 and Tween 80, on the rate of DBT desulfurization by *R. erythropolis* was also investigated. Triton X-100 completely inhibited biodesulfurization of DBT and growth of *R. erythropolis* Q1a-22 at concentrations greater than and equal to its critical micelle concentration, CMC, 1.7×10^{-4} M. This result shows that Triton X-100 is toxic to *R. erythropolis* Q1a-22 especially at concentrations above its CMC. The critical micelle concentration (CMC) refers to the concentration at which micelles first form in solution. It is sharply defined for each surfactant and marked by quite sharp changes in the equilibrium or transport properties of the surfactant solution [7].

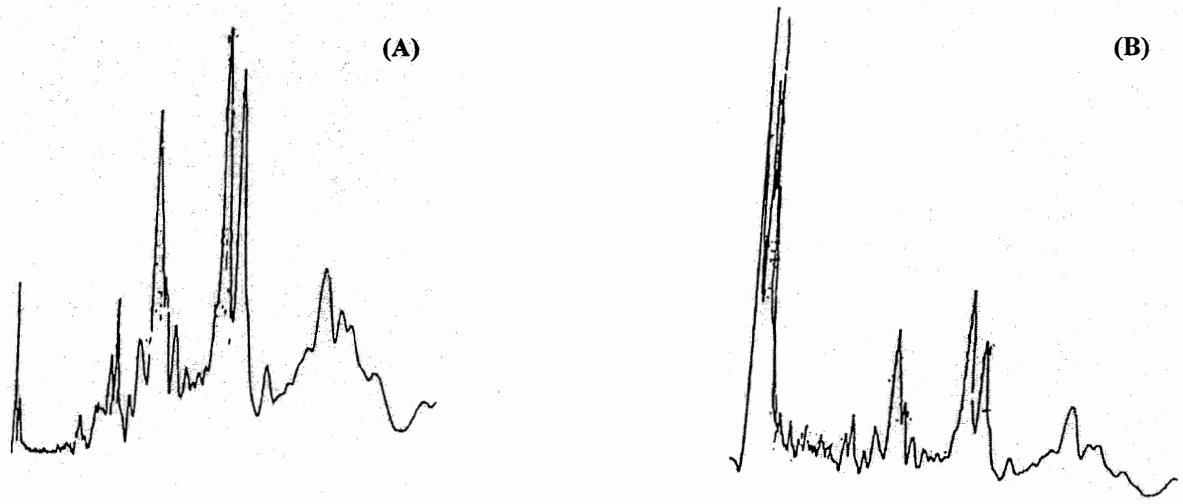

Fig. 7. HPLC chromatogram of petroleum (A) *without R. erythropolis Q1a-22 and* (B) *with R. erythropolis Q1a-22.*

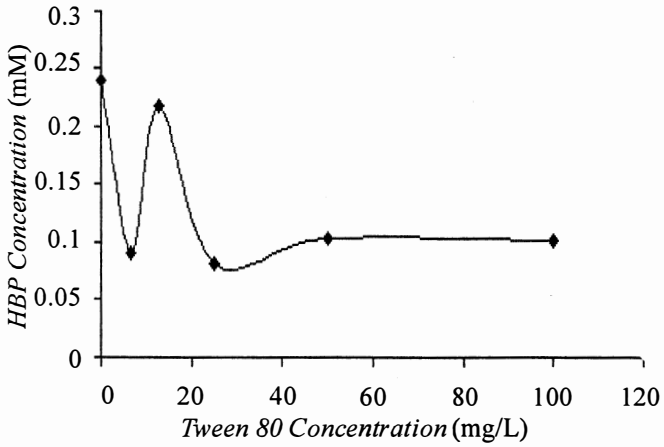

Fig. 8. Effect of nonionic surfactant, Tween 80, on the biodesulfurization of 0.54 mM DBT by R. erythropolis Q1a-22.

The data obtained from HPLC analysis of those samples with Tween 80, unlike Triton X-100, showed no form of toxicity towards *R. erythropolis* Q1a-22. Growth of the bacteria was still observed even at concentrations above its CMC, 13 mg/L. Maximum desulfurization activity was observed at samples with Tween 80 at its CMC (Fig. 8). This clearly shows that in the presence of non-toxic surfactants there is an increase in the bioavailability of DBT at the critical micelle concentration, wherein equilibrium between the formation of micelles and the separation of a surfactant monomer from the micelle is taking place. However, for this study, higher extent of desulfurization was still observed for those samples without the added surfactant. The presence of surfactant during may have some significant effect during desulfurization of crude-oil derived fuels in terms of the availability of these fuels for biodesulfurization.

CONCLUSION

Biodesulfurization as an alternative technique or as a follow up process to the high temperature/pressure commercial process undoubtedly shows great potential. From the studies made on DBT and petroleum samples, *R. erythropolis* Q1a-22 was proven to be the most effective strain for biodesulfurization following the 4S DBT degradation pathway proposed by Kilbane. Thus, its application for petroleum biodesulfurization deserves high consideration. This study also explored the feasibility of applying nonionic surfactants to biodesulfurization cultures of DBT since surfactants have found applications in the field of environmental microbiology. The data obtained showed that the effect of nonionic surfactants on the biodesulfurization activity of *R. erythropolis* Q1a-22 on DBT is not general but rather specific for each type of surfactant and its interaction with the microorganism.

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