Isolation and characterization of an SDS-degrading *Klebsiella oxytoca*

M.Y. Shukor*, W.S.W. Husin, M.F.A. Rahman, N.A. Shamaan and M.A. Syed

Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia,
UPM 43400 Serdang, Selangor, Malaysia

(Received: December 18, 2007; Revised received: June 10, 2008; Accepted: July 20, 2008)

Abstract: Sodium dodecyl sulfate (SDS) is one of the main components in the detergent and cosmetic industries. Its bioremediation by suitable microorganism has begun to receive more attention as the amount of SDS usage increases to a point where treatment plants would not be able to cope with the increasing amount of SDS in wastewater. The purpose of this work was to isolate local SDS-degrading bacteria. Screening was carried out by the conventional enrichment-culture technique. Six SDS-degrading bacteria were isolated. Of these isolates, isolate S14 showed the highest degradation of SDS with 90% degradation after three days of incubation. Isolate S14 was tentatively identified as *Klebsiella oxytoca* strain DRY14 based on carbon utilization profiles using Biolog GN plates and partial 16S rDNA molecular phylogeny. SDS degradation by the bacterium was optimum at 37°C. Ammonium sulphate; at 2.0 g l\(^{-1}\), was found to be the best nitrogen source for the growth of strain DRY14. Maximum growth on SDS was observed at pH 7.25. The strain exhibited optimum growth at SDS concentration of 2.0 g l\(^{-1}\) and was completely inhibited at 10 g l\(^{-1}\) SDS. At the tolerable initial concentration of 2.0 g l\(^{-1}\), almost 80% of 2.0 g l\(^{-1}\) SDS was degraded after 4 days of incubation concomitant with increase in cellular growth. The K\(_{\text{m}}\) and V\(_{\text{max}}\) values calculated for the alkylation action from this bacterium were 0.1 mM SDS and 1.07 μmol min\(^{-1}\) mg\(^{-1}\) protein, respectively.

Key words: Sodium dodecyl sulfate, Biodegradation, *Klebsiella oxytoca*

PDF of full length paper is available with author (*yunus@biotech.upm.edu.my*)

Introduction

Wastewater problem due to excessive use of detergents is becoming more pressing as detergents are known to have detrimental effects to aquatic life (Chukwu and Odunze, 2006; Kumar et al., 2007). Of the massive amounts of SDS and other surfactants used domestically, it is likely that most of it end up in wastewater flows (Liwarska-Bizukojc et al., 2005). SDS and other surfactants have been considered to be biodegradable by aerobic processes (Cserehi et al., 2002). However, the mass loadings indicated that even at these removal ratios, appreciable amounts are released into receiving waters where a variety of surfactants was identified in both surface and drinking water extracts (Isobe et al., 2004). According to literature data, anionic surfactants give toxic effects to various aquatic organisms at concentrations as low as 0.0025 mg l\(^{-1}\) (Peterson et al., 2000). In Malaysia, the pollution cause by the use of detergent is expected to rise steadily. In the year 2000 alone Malaysia imported a total of 14,625 ton of anionic surfactants valued at Malaysian Ringgit 57.5 million (Ahmed et al., 2002). Due to this, there is a need to isolate more SDS-degrading bacteria to cope for an ever-growing pollution of this detergent. Biodegradation of anionic surfactant by anaerobic conditions of *Pseudomonas* sp. strain C12B was among the first to be studied (Payne and Feisal, 1963). The soil temperature in Malaysia, being a tropical climate, can be as high as 35°C year round (Sinnakannu et al., 2004). This poses a problem if bioremediation strains reported in the literature are to be used since many of the strains have optimum growth temperature of 30°C and less (Abboud et al., 2007). In order to design effective bioremediation strategy in the tropics, efficient strains having fast degradation capacity and high optimum growth temperatures are much needed. In this work we report on the isolation and characterization of a local SDS-degrading bacterium isolated from soil with an optimum growth temperature using SDS as a carbon source at 37°C. The isolate was able to degrade approximately 80% of 2.0 g l\(^{-1}\) SDS after 4 days of incubation concomitant with increase in cellular growth. The characteristics of this isolate suggest that it would be useful in the bioremediation of SDS in the tropics.

Materials and Methods

Isolation of bacteria: Samples were collected from soils and water contaminated with detergent from a car wash outlet in Serdang, Selangor. Soil samples were taken randomly to a depth of 5 cm from the topsoil using sterile spatula and stored in sterile screw-capped polycarbonate tubes. The outlet has detergent concentration measured using the methylene blue active substance (MBAS) assay ranging from 300 to 1000 mg l\(^{-1}\) equivalent SDS. Five ml of the water samples were aseptically inoculated into 45 ml sterile SDS enrichment media in a 250 ml conical flask. The cultures were incubated at room temperature (25°C) with shaking at 150 rpm on orbital shaker in basal salts (BS) medium containing (gram per liter) of the following: KH\(_2\)PO\(_4\), 1.36; Na\(_2\)HPO\(_4\), 1.388; MgSO\(_4\), 0.01; CaCl\(_2\), 0.01; (NH\(_4\))\(_2\)SO\(_4\), 7.7. The medium also contained the following trace elements containing (gram per liter): ZnSO\(_4\)·7H\(_2\)O, 0.01; MnCl\(_2\)·4H\(_2\)O, 0.01; H\(_2\)BO\(_3\), 0.01; CoCl\(_2\)·6H\(_2\)O, 0.01; FeSO\(_4\)·7H\(_2\)O, 0.01; CuCl\(_2·2H\_2O), 0.01; Na\(_2\)MoO\(_4·2H\_2O), 0.01. The medium was supplemented with filter-sterilized SDS as a carbon source with a final concentration of 1.0 g l\(^{-1}\) (Dhouib et al., 2003). Six bacterial
isolates were selected from the preliminary screening based on growth on 1.0 g l⁻¹ SDS as a sole carbon source using MBAS assay. Identification at species level was performed by using Biolog GN MicroPlate (Biolog, Hayward, CA, USA) according to the manufacturer’s instructions and molecular phylogenetics studies.

Methylene blue active substance (MBAS) assay: The concentrations of residual SDS were determined by measuring the intensity of methylene blue in a chloroform extraction process (Hayashi, 1975). One hundred microlitres of samples were added into 100 ml separating funnel containing 9.9 ml deionized H₂O followed by the addition of 2.5 ml methylene blue solution and 1 ml of chloroform. The funnel was shaken vigorously for 15 seconds and the mixture was let to separate and settle. The chloroform layer was drawn off into a second funnel. The extraction was repeated three times using 1 ml chloroform each time. All chloroform extracts were combined in the second funnel before adding 5.0 ml of wash solution. The funnel was then shaken vigorously for 15 seconds. The chloroform layer was drawn off into a 10 ml volumetric flask. The wash solution was extracted twice with 1 ml chloroform. All extracts were combined and diluted to the 10-ml mark with chloroform. The absorbance was read at 652 nm against blank chloroform in a quartz cuvette.

16S rDNA gene sequencing and phylogenetic analysis: Genomic DNA was extracted from bacterial colonies by alkaline lysis. PCR amplification was performed using Biometra T Gradient PCR (Montreal Biotech Inc., Kirkland, QC). The PCR mixture contained 0.5 μl of each primer, 200 μM of each deoxynucleotide triphosphate, 1X reaction buffer, 2.5 U of Taq DNA polymerase (Promega) to achieve a final volume of 50 μl. The 16S rDNA gene from the genomic DNA was amplified by PCR using the following forward and reverse primers of 16S rDNA: 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCACCAGCCGA-3' (Devereux and Wilkinson, 2004). PCR was performed under the following conditions: initial denaturation at 94°C for 3 min; 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 10 min. Cycle sequencing was subsequently performed with the Big Dye terminator kit (Perkin-Elmer Applied Biosystems) as recommended by the manufacturer. The resultant 1449 bases were compared with the GenBank database using the Blast server at NCBI (Altschul et al., 1990). This analysis showed this sequence to be closely related to strain DRY14 deposited in GenBank under the accession number DQ226215.

Phylogenetic analysis: A multiple alignment of 17 16S rDNA gene sequences closely matches strain DRY14 were retrieved from GenBank and were aligned using ClustalW (Thompson et al., 1994). A phylogenetic tree was constructed by using PHYLIP, version 3.573 (Retief, 2000), with E. coli as the outgroup in the cladogram. Evolutionary distance matrices for the neighbour-joining and UPGMA method were computed using the DNADIST algorithm program. The model of nucleotide substitution is those of Jukes and Cantor (1969). Phylogenetic tree was inferred by using the neighbour-joining method of Saitou and Nei (1987). With each algorithm, confidence levels for individual branches within the tree were checked by repeating the PHYLIP analysis with 1000 bootstraps (Felsenstein, 1985) by the SEQBOOT program in the PHYLIP package. Majority rule (50%) consensus trees were constructed using the CONSENSE program (Margush and McMorris, 1981) and the tree was viewed using TREEVIEW (Page, 1996).

Preparation of enzyme extracts: Bacterial cells were harvested at day 2 by centrifuging a 2 l culture at 10,000 x g for 20 min at 4 °C in a Beckman J20 high-speed centrifuge. Cell pellets were resuspended in 10 mM Tris-HCl (pH 7.5). The cells were ruptured by sonication with a sonicator (Biosonic 111™, Bronwill Scientific, Rochester, N.Y) for a total duration of 30 min, consisting of intermittent sonication for 30 s on and 30 s off. The crude fraction was subjected to ultracentrifugation at 105,000 x g for 2 hr and the supernatant taken for enzyme studies. Protein concentration was determined by Coomassie dye-binding assay at 595 nm using the Bio-Rad™ Bradford reagent.

Alkylsulfatase assay: Alkylsulfatase activity in cell extracts was assayed by incubating 50 μl partially purified enzyme (0.14 mg ml⁻¹ protein) of isolate S14 with 450 μl of 50 mM Tris-HCl and 500 μl of 100 mM SDS. The loss of the substrate was measured by MBAS assay as described before. The disappearance of SDS was linear until 15 min under the assay conditions. Total enzyme activity was determined from the initial rates of SDS disappearance. One unit of enzyme was defined as the amount of enzyme which converted 1 µmol of SDS per minute under assay conditions.

Statistical analysis: Values are means ± SE of at least three replicates. All data were analyzed using Graphpad Prism version 3.0 and Graphpad InStat version 3.05. Comparison between groups was performed using a Student’s t-test or a one-way analysis of variance with post hoc analysis by Tukey’s test (Miller and Miller, 2005). p < 0.05 was considered statistically significant.

Results and Discussion

Isolation of SDS-degrading bacteria: Out of the six SDS-degrading bacteria isolated, isolate S14 showed significantly the highest degradation of SDS (p<0.05) with 90% degradation after three days incubation (Fig. 1). Isolates S1, S5 and S8 exhibited lower degradation of SDS with only 60%, 50% and 44% degradation respectively. Isolates S4 and S7 gave the lowest percentage of degradation with only 24% and 17% respectively. Control using uninculated medium showed no degradation within the same period of time.

Identification of the isolate: Isolate S14 is a gram negative bacterium. The colony was creamy white in appearance. Under magnification, the bacterium appeared rod-like existing as single or in pairs. A moderate bootstrap value (43.2%) linked strain DRY14 to Klebsiella oxytoca (Fig. 2) indicating a moderately strong phylogenetic relationship of the strain to this species. The strain was further grouped
Isolation and characterization of an SDS-degrading Klebsiella oxytoca

Fig. 2: A phylogram (neighbour-joining method) showing genetic relationship between isolate DRY14 and other related reference microorganisms based on the 16S rRNA gene sequence analysis. Species names are followed by the accession numbers of their 16S rRNA sequences. The numbers at branching points or nodes refer to bootstrap values, based on 1000 re-samplings. Scale bar represents 100 nucleotide substitutions. Bacillus subtilis strain KT1003 is the outgroup.

Fig. 1: Degradation of SDS by soil isolates S1 (○), S4 (■), S5 (▲), S7 (○), S8 (△) and S14 (○) in comparison to control (●). Data represent means ± SEM, n=3.

The effects of nitrogen source on growth: From the results obtained, ammonium sulphate gave the significantly highest growth as a nitrogen source for the growth of strain DRY14 compared to other carbon sources (p<0.05). This was followed by sodium nitrite and finally sodium nitrate (Fig. 3). The effects of different concentrations of ammonium sulphate in the range of 0-10 g l⁻¹ was evaluated. The results showed that this isolate required 2.0 g l⁻¹ ammonium sulphate for optimum growth. Growth was rapidly reduced when the concentrations of the nitrogen source was further increased. Ammonium sulphate was reported to be the best nitrogen source for SDS degradation by Citrobacter braakii (Dhouib et al., 2003). In contrast, Comamonas terrigena showed optimum growth with ammonium nitrate as the nitrogen source (Roig et al., 1998). A more complex nitrogen source in the form of nutrient broth has been used to enhance SDS-degradation by a bacterial consortium. However, the use of this complex nitrogen source did pose SDS-degrading enzyme induction problem (Abboud et al., 2007) and this suggests that a balance must be found between enhancement of bacterial growth and the goal of SDS-degradation in future studies. Identification of the best nitrogen source and its optimum concentration for growth would help in designing effective bioremediation strategy for surfactant contamination (Frischke and Hofrichter, 1999).

The effects of SDS concentrations on growth: The effect of SDS as a carbon source to growth of isolate DRY14 was studied using SDS concentrations of up to 10.0 g l⁻¹. The isolate exhibited an increase in cellular growth as SDS concentrations was raised culminating to an optimum SDS concentration of between 1.0 and 2.0 g l⁻¹ (Fig. 4). The bacterial growth decreased dramatically from 2.0 g l⁻¹ to 10.0 g l⁻¹. SDS as the sole source of carbon is needed in large quantities as carbon is the basic structural unit of all organic compounds. However, at certain concentrations, SDS can be toxic to microorganisms. This is because, in microorganisms, SDS adsorption produces a depolarization of cell membrane and consequently decreases the absorption of nutrient and modifies the release of substance from cell metabolism. The bacteria may also be

Journal of Environmental Biology  January, 2009
killed by the possible detergent effects which strip the lipopolysaccharide outer layer of Gram negative bacteria when the SDS concentration is high (Odahara, 2004; Abboud et al., 2007). Eventually, the viability of microorganisms will decrease (Liwarska-Bizukojc et al., 2005). The result from this work shows that this isolate required 2.0 g l\(^{-1}\) of SDS for optimum growth. Previous study showed that *Citrobacter braakii* demonstrated optimum growth at surfactant concentration of 1.0 g l\(^{-1}\) (Dhouib et al., 2003). Bacterial growth on higher SDS concentration at 4 g l\(^{-1}\) has been reported (Abboud et al., 2007). To date, the most tolerant SDS-degrading bacterium is *Pseudomonas* strain CL12B that could grow optimally on 0.025 M SDS or 7.2 g l\(^{-1}\) (Payne and Feisal, 1963). In all cases, growth dramatically decreases at higher concentrations of SDS.

**Optimization of pH:** The effect of pH to the growth of isolate DRY14 on 2.0 g l\(^{-1}\) SDS was studied using an overlapping buffer system consisting of phosphate and carbonate (50 mM) spanning the pH range from 6.5 to 8.5. The isolate gave significantly the highest growth at pH 7.25 both in phosphate and carbonate buffers at the same pH (p<0.05) (Fig. 5) with growth on phosphate buffer giving significantly higher growth than on carbonate buffer (p<0.05). Growth was dramatically reduced at lower or higher pHs than the optimum. The preference to neutrality or near neutrality in terms of optimal growth on SDS is shared by many other SDS-degrading bacteria such as *Citrobacter braakii* at pH 7.0 (Dhouib et al., 2003), *Comamonas terrigena* strain N3H at pH 7.4 (Roig et al., 1998) and pH 7.5 to 8.0 for *Pseudomonas* strain C12B (Payne and Feisal, 1963). In another report, growth on SDS by a novel consortium of *Acinetobacter calcoaceticus* and *Pantoea agglomerans* required pH 8.5 for efficient degradation (Abboud et al., 2007). In bioremediation works, a cheap source of pH controlling chemical such as calcium carbonate can be added to soil during bioremediation to achieve near neutrality in order to optimize remediation (Johnston et al., 1996).

**Optimization of temperature:** The effect of temperature on the cellular growth of strain DRY14 on 2.0 g l\(^{-1}\) SDS was studied at temperatures ranging from 10 to 50°C. Celluar growth was increased as the temperature was increased from 10°C reaching an optimum at 37°C before a dramatic decline in growth is seen at higher
Isolation and characterization of an SDS-degrading Klebsiella oxytoca strain DRY14 after growth optimizations. Data represents mean ± SEM, n=3

Fig. 6: The effect of temperatures on the growth of strain DRY14. Data represent means ± SEM, n=3

Fig. 7: SDS degradation (●), abiotic control (○) and cellular growth (■) of strain DRY14 after growth optimizations. Data represents mean ± SEM, n=3

The optimum temperature that supports growth at 37°C is quite high compared to many other mesophilic SDS-degrading bacteria reported in the literature. Citrobacter braakii, Delftia acidovorans strain SPB1, Pseudomonas strain C12B, Acinetobacter calcoaceticus and Pantoea agglomerans all required 30°C for optimal SDS degradation (Payne and Feisal, 1963; Schulz et al., 2000; Dhouib et al., 2003; Abboud et al., 2007) while Comamonas terrigena strain N3H showed optimum growth at 28°C (Roig et al., 1998). Marchesi et al. (1997) reported a lower temperature for the degradation of SDS by Pseudomonas sp. at 25°C. Since soils in Malaysia could reach temperatures as high as 35°C (Sinnakkannu et al., 2004), isolate DRY14 is a suitable indigenous bacteria that could be employed in soil bioremediation of surfactant.

SDS degradation study: The degradation of SDS together with cellular growth using the optimum conditions obtained in the optimization studies is shown in Fig. 7. Almost 80% of 2.0 g l⁻¹ SDS was degraded after 4 days of incubation concomitant with increase in cellular growth. Ten days of incubation were needed to degrade SDS completely. Growth then enters plateau on days 8 onwards, possibly due to substrate depletion. The SDS content remained unchanged in the uninoculated control flasks showing that no abiotic elimination of SDS occurred. Hence, SDS degradation in the inoculated flasks is attributed completely to microbial degradation. Disappearance of bubbles during incubation period also indicates the absence of SDS due to degradation of this surfactant from the culture. A much higher degradation of SDS at 4.0 g l⁻¹ SDS has been reported using a consortium of Acinetobacter calcoaceticus and Pantoea agglomerans with complete degradation occurring after approximately 5 days. However the conditions of degradation are markedly different from our works with the usage of nutrient broth as a supplement and agitation at high speed (250 rpm) in the study using the consortium (Abboud et al., 2007).

Alkysulfatase activity: The effect of substrate concentration on the activity of alkysulfatase was carried out by performing standard assays at varying concentration of SDS. Alkysulfatase produced a typical Michaelis-Menten curve when reacted with SDS. The Michaelis-Menten and Lineweaver-Burke plots for alkysulfatase with SDS as the substrate were constructed to calculate the apparent $K_m^{(app)}$ and the apparent Michaelis-Menten constant and $V_{max}^{(app)}$, apparent maximum velocity. The $K_{m}^{(app)}$ and $V_{max}^{(app)}$ values calculated from Lineweaver-Burke plot gave values of 0.1 mM SDS and 1.07 µmol min⁻¹mg⁻¹ protein, respectively. Lillis et al. (1983) reported that the $K_m$ and $V_{max}$ of alkysulfatase from Pseudomonas putida FLA calculated from Lineweaver-Burke plot were higher with 1.0 mM and 9.0 µmol SDS per minute, respectively. Alower $K_m$ value of 0.034 mM was demonstrated by Pseudomonas putida S-313 (Kahnert and Kertesz, 2000).

In conclusion, we have isolated an SDS-degrading bacterium from an SDS-polluted water sample from Malaysia. Growth optimization studies of the isolate were performed on various physicochemical parameters for promoting higher degradation rate so that the optimized parameters can be applied on the field. The isolate exhibited mixed result in terms of growth optimizations using SDS as the carbon source compared to other published works. The relatively high optimum temperature for growth on SDS exhibited by this bacterium is suitable to be used for the bioremediation of SDS-polluted sites in Malaysia. Work is underway to study heavy metals resistant of the strain since it is well known that some environmental samples contain significant amount of heavy metals which could
inhibit bioremediation. We are also in the process of characterizing the enzymes and genes involved in SDS-degradation and working on immobilization studies using this bacterium to remediate SDS-contaminated water bodies.

References