



## Characterization of a sodium dodecyl sulphate-degrading *Pseudomonas* sp. strain DRY15 from Antarctic soil

M.I.E. Halmi<sup>1</sup>, W.S.W. Hussin<sup>1</sup>, A. Aqlima<sup>1</sup>, M.A. Syed<sup>1</sup>, L. Ruberto<sup>2</sup>, W.P. MacCormack<sup>2</sup> and M.Y. Shukor<sup>1\*</sup>

<sup>1</sup>Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia 43400 UPM Serdang, Selangor, Malaysia

<sup>2</sup>Instituto Antartico Argentino, Cerrito 1248 (1010), Buenos Aires, Argentina

\*Corresponding Author email : yunus@biotech.upm.edu.my

### Publication Info

Paper received:  
04 December 2012

Revised received:  
07 May 2013

Accepted:  
01 June 2013

### Abstract

A bacterium capable of biodegrading surfactant sodium dodecyl sulphate (SDS) was isolated from Antarctic soil. The isolate was tentatively identified as *Pseudomonas* sp. strain DRY15 based on carbon utilization profiles using Biolog GN plates and partial 16S rDNA molecular phylogeny. Growth characteristic studies showed that the bacterium grew optimally at 10°C, 7.25 pH, 1 g l<sup>-1</sup> SDS as a sole carbon source and 2 g l<sup>-1</sup> ammonium sulphate as nitrogen source. Growth was completely inhibited at 5 g l<sup>-1</sup> SDS. At a tolerable initial concentration of 2 g l<sup>-1</sup>, approximately 90% of SDS was degraded after an incubation period of eight days. The best growth kinetic model to fit experimental data was the Haldane model of substrate inhibition with a correlation coefficient value of 0.97. The maximum growth rate was 0.372 hr<sup>-1</sup> while the saturation constant or half velocity constant (K<sub>s</sub>) and inhibition constant (K<sub>i</sub>), were 0.094% and 11.212 % SDS, respectively. Other detergent tested as carbon sources at 1 g l<sup>-1</sup> was Tergitol NP9, Tergitol 15S9, Witconol 2301 (methyl oleate), sodium dodecylbenzene sulfonate (SDBS), benzethonium chloride, and benzalkonium chloride showed Tergitol NP9, Tergitol 15S9, Witconol 2301 and the anionic SDBS supported growth with the highest growth exhibited by SDBS.

### Key words

Antarctica, Biodegradation, *Pseudomonas* sp., SDS

### Introduction

The setting up of many bases and increase in the numbers of tourists and tourist's ships in the cold region has introduced anthropogenic pollutants in Antarctica. Anionic surfactant such as sodium dodecyl sulphate (SDS) has been reported to occur in the Antarctic Maxwell Bay and its adjacent sea areas at concentrations up to 1.0 mg l<sup>-1</sup> (Junfeng *et al.*, 1998) and biodegradation of the surfactant by sea water bacteria has been reported (George, 2002). Detergents are known to have detrimental effects on aquatic life (Liwarska-Bizukoje *et al.*, 2005; Chukwu and Odunzeh, 2006; Kumar *et al.*, 2007). Pettersson *et al.*, 2000 reported that anionic surfactants produce effects on various aquatic organisms at concentrations from 0.0025 to 300 mg l<sup>-1</sup>. Another study indicated that exposure to SDS

has a detrimental effect on oyster digestive gland, resulting in the perturbation of the metabolic and nutritional functions, and having a direct influence on oyster survival (Rosety *et al.*, 2000). Toxicity towards invertebrates and crustaceans could occur as the considerable amount of anionic surfactants released into water. The life cycle of aquatic animals has also been influenced by the anionic surfactants, modified the behavior of the fish such as erratic movements, muscle spasms and body torsion (Cserhati *et al.*, 2002). Due to this, remediation of SDS is of vital importance. Microbes are known for their ability to degrade organics including SDS (Pant *et al.*, 2008; Shukor *et al.*, 2009), and their use as bioremediating agents is important for economical removal of xenobiotic pollutants. Biodegradation of anionic surfactant under aerobic conditions was first studied in the bacterium *Pseudomonas*

sp. strain C12B (Payne and Feisal, 1963), and to date quite a number of SDS-degrading bacteria have been isolated and characterized (Roig *et al.*, 1998; Schulz *et al.*, 2000; Dhouib *et al.*, 2003; Chaturvedi and Kumar, 2010; 2011a; 2011b). Works on cold-adapted microbes with ability to degrade SDS are rare, and were first reported by Margesin and Schinner (1998). In the present study, isolation and characterization of the surfactant-degrading bacterium from Antarctica is reported.

### Materials and Methods

**Isolation of bacteria :** Soil and water samples were collected 15-20 cm beneath the surface from Jubany Station, an Argentinean Base on King George Island, South Shetlands Islands, Antarctica (61.5°S; 54.55°W) from December 2002 to January 2003 in sterile screw-capped vials. The samples were immediately placed in a freezer and stored at -20°C until returned to the laboratory for further examination. Five grams of soil or five ml of water samples were aseptically inoculated into 45 ml sterile SDS enrichment media in a 250 ml conical flask, and incubated at 10 °C with shaking at 150 rpm on an orbital shaker. The basal salts medium contained the followings:  $\text{KH}_2\text{PO}_4$ , (1.36 g l<sup>-1</sup>),  $\text{Na}_2\text{HPO}_4$ , (1.39 g l<sup>-1</sup>),  $\text{KNO}_3$ , (0.5 g l<sup>-1</sup>),  $\text{MgSO}_4$  (0.01 g l<sup>-1</sup>),  $\text{CaCl}_2$  (0.01 g l<sup>-1</sup>) and  $(\text{NH}_4)_2\text{SO}_4$  (7.7 g l<sup>-1</sup>). The medium also contained the following trace elements:  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.01 g l<sup>-1</sup>),  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (0.01 g l<sup>-1</sup>),  $\text{H}_3\text{BO}_4$  (0.01 g l<sup>-1</sup>),  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (0.01 g l<sup>-1</sup>),  $\text{FeSO}_4 \cdot 2\text{H}_2\text{O}$  (0.01 g l<sup>-1</sup>),  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  (0.01 g l<sup>-1</sup>) and  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (0.01 g l<sup>-1</sup>). Filter-sterilized sodium dodecyl sulphate was added to the medium as a carbon source at the final concentration of 1.0 g l<sup>-1</sup> (Dhouib *et al.*, 2003). After several series of subcultures, 1 ml sample was taken from the final enrichment flask, serially diluted and plated on solid sodium dodecyl sulphate agar plate, and incubated at 10°C for 10 days. A pure sodium dodecyl sulphate -degrading bacterial colony was obtained.

**Assay of methylene blue active substance (MBAS):** In brief, the concentration of residual SDS was determined by measuring the intensity of methylene blue in chloroform extraction (Hayashi, 1975). 100 ml of samples was added into 100 ml separating funnels containing 9.9 ml of deionized H<sub>2</sub>O followed by the addition of 2.5 ml methylene blue solution and 1 ml of chloroform. The funnel was shaken vigorously for 15 sec and the mixture was left to separate and settle. The chloroform layer was drawn off into a second funnel and the extraction was repeated thrice using 1 ml of chloroform each time. All chloroform extracts were then combined in the second funnel before the addition of 5.0 ml of wash solution followed by vigorous shaking of the funnel for 15 sec. The chloroform layer was drawn off in a 10 ml volumetric flask and the wash solution was extracted twice with 1 ml chloroform. All extracts were then combined and

diluted to the 10 ml mark with chloroform. The absorbance was read at 652 nm against blank chloroform.

Identification at species level was performed by using Biolog GN MicroPlate (Biolog, Hayward, CA, USA) and molecular phylogenetics studies. A pure culture of the bacterium was grown on a Biolog Universal Growth agar plate. Bacteria were then swabbed off from the surface of the agar plate and suspended to a specified density in GN Inoculating Fluid. 150 µl of a bacterial suspension was then pipetted into the GN microplate and was at 30° or 35°C depending upon the nature of the organism for 4-24 hr. The plate was then read with the Biolog Micro Station TM System and compared to database. Biolog GN gave identification to the *Pseudomonas* genus but with low probability (<95%). Genomic DNA extraction, PCR of the 16S rDNA and comparison of the partial sequence obtained (1448 base pairs) with the GenBank database using the Blast server at NCBI (Altschul *et al.*, 1990) showed that the sequence to be 99% similar to *Pseudomonas* sp. The 16S rRNA ribosomal gene sequence for this isolate have been deposited in GenBank under the following accession number DQ226216. At this juncture, the isolate is assigned tentatively as *Pseudomonas* sp. strain DRY15.

**Kinetic studies :** The profile of various biomass growth rates were used in obtaining kinetic parameters from batch experiments. Bacterial dry weight determination was carried by plotting dry weight of biomass per liter against bacterial count (CFU ml<sup>-1</sup>). The values of the specific growth rate coefficient  $\mu$  at each initial diesel concentration were obtained by plotting  $\ln X$  (bacterial dry weight) vs. time. These values were plotted against substrate concentrations to obtain a nonlinear curve for modeling. The classical Monod model is often used when substrate is not inhibiting growth while the Haldane model is the model of choice in modeling growth under substrate inhibition conditions. The constants were obtained by running data on graphPad Prism using the Michaelis-Menten substrate inhibition kinetics and replacing the resultant enzyme kinetic constants with SDS degradation kinetics constants.

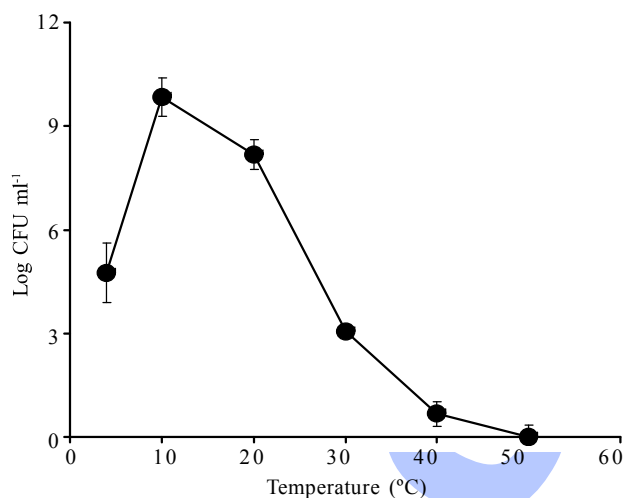
**Statistical analysis :** All data were analyzed using Graphpad Prism version 3.0 and Graphpad InStat version 3.05. Values are means  $\pm$  SE. Comparison between groups was performed using a Student's t-test or a one-way analysis of variance with post hoc analysis by Tukey's test (Miller and Miller, 2000).  $P < 0.05$  was considered statistically significant.

### Results and Discussion

**Effect of temperature :** The study of optimum temperature required for the growth of microbes would be very useful for bioremediation purposes especially with bioaugmentation

since large-scale growth of the bacteria can be carried out under closed optimum conditions (King *et al.*, 1992). In the present study, growth on SDS was significantly highest at 10°C ( $p < 0.05$ ). Growth dropped rapidly at incubation temperatures above 20°C and almost no growth occurred at temperatures higher than 40°C (Fig. 1) suggesting that the isolate is a typical psychrotolerant bacterium. The result in this study is very similar to the works of Margesin and Schinner (1998) on psychrotolerant SDS degrading bacterial consortia from alpine regions. The low temperature requirement for bacterial growth means that the bacterium is suitable for bioremediation of SDS at Polar regions.

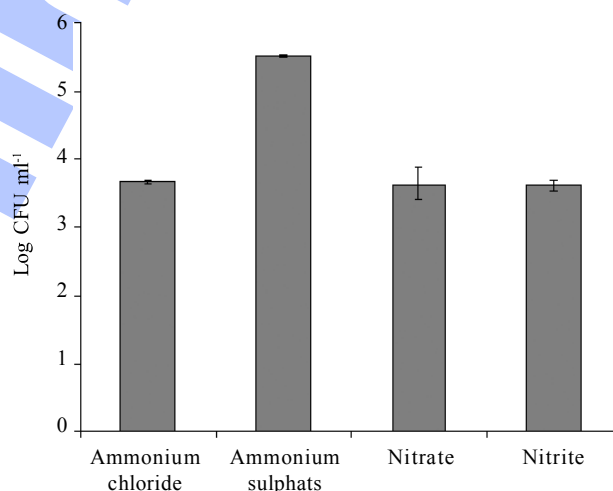
**Effect of nitrogen source on growth :** Nitrogen source is one of the important factors that affect the growth of bacteria. Hence, identification of the best nitrogen source and its optimum concentration for growth would help in designing effective bioremediation strategy (Fritsche and Hofrichter, 1999). Ammonium sulphate significantly gave the highest growth on SDS compared to other nitrogen sources ( $p < 0.05$ ) (Fig. 2). Ammonium sulphate has been widely known as a good nitrogen source for biodegradation media. Ammonium sulphate was reported to be the best nitrogen source for SDS degradation (Dhouib *et al.*, 2003, Shukor *et al.*, 2009; Chaturvedi and Kumar, 2011a; 2011b). *Comamonas terrigena* strain N3H showed optimum growth with ammonium nitrate as the nitrogen source (Roig *et al.*, 1998). Optimum growth was achieved at 2 g l<sup>-1</sup>. Other surfactant degraders like *Citrobacter braakii* required 7.7 g l<sup>-1</sup> ammonium sulphate (Dhouib *et al.*, 2003) whereas *Comamonas terrigena* strain N3H showed an optimum growth with 5.4 g l<sup>-1</sup> ammonium nitrate (Roig *et al.*, 1998).



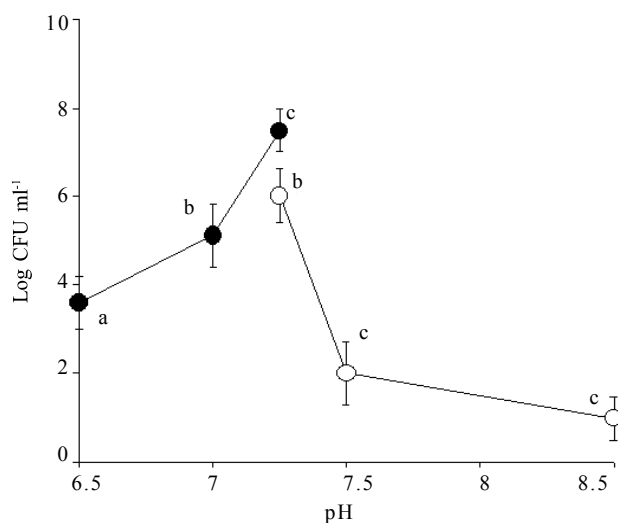
**Fig. 1 :** The effect of temperatures on the growth of strain DRY15. Data is mean standard error (n=3). Values with the same letter are not significantly different ( $p > 0.05$ )

**Effect of pH :** Maintenance of pH in bacterial medium is important since pH strongly affects bacterial growth. Identification of the pH optima for growth would help in designing effective bioremediation strategy (Davey, 1994). The highest significant cellular growth on SDS was found at pH 7.25 (Fig. 3). The preference for neutral to slightly alkaline pH for the degradation of SDS is demonstrated by several other SDS-degrading bacteria. *Klebsiella oxytoca* strain DRY14 exhibited an optimum growth at pH 7.25 (Shukor *et al.*, 2009). Where *Delftia acidovorans* strain SPB1 showed an optimum growth on SDS at 7.2 pH (Schulz *et al.*, 2000). *Citrobacter braakii* required pH 7.0 (Dhouib *et al.*, 2003), *Comamonas terrigena* strain N3H required pH 7.4 (Roig *et al.*, 1998), *Pseudomonas aeruginosa* MTCC 10311 showed maximum growth at pH 7.5 (Ambily and Jisha, 2012) while *Pseudomonas nitroreducens* (MTCC 10463) and *Pseudomonas aeruginosa* (MTCC 10462) grew optimally in between pH 7.0 and 7.5 (Asok and Jisha, 2012). The growth of strain DRY15 decreased significantly at pH 9.5, presumably due to extreme alkaline conditions.

**Effect of SDS concentrations on growth :** SDS as the sole source of carbon is required in large quantities as carbon is the basic structural unit of all organic compounds. The bacteria may also be killed by stripping of the lipopolysaccharide outer layer of Gram negative bacteria when SDS concentration is high (Odahara, 2004). Eventually, the viability of microorganisms will decrease (Liwarska-Bizukojc *et al.*, 2005). Strain DRY15 showed significantly highest growth ( $p < 0.05$ ) at SDS concentration of 1.0 g l<sup>-1</sup>. The bacterium exhibited lower growth at SDS concentrations between 2 and 3 g l<sup>-1</sup> and was completely inhibited at 5 g l<sup>-1</sup> (Fig. 4). A tropical isolate, *Klebsiella*



**Fig. 2 :** The effect of nitrogen sources on the growth of strain DRY15. Data is mean standard error (n=3). Values with the same letter are not significantly different ( $p > 0.05$ )

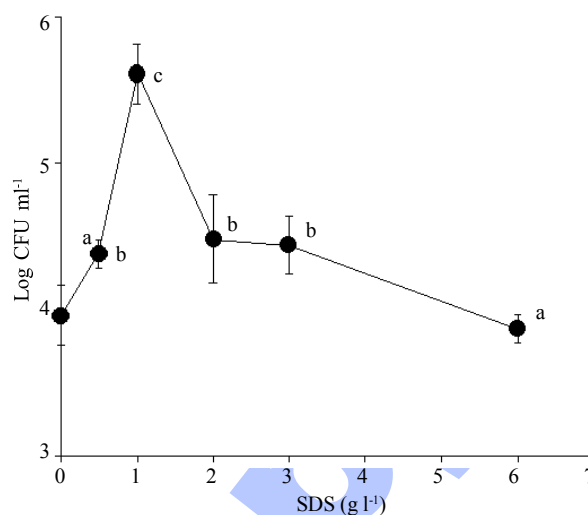


**Fig. 3 :** The effect of pH on the growth of strain DRY15 using an overlapping buffer system consisting of phosphate (○) and carbonate (●). Data is mean standard error (n=3). Values with the same letter are not significantly different ( $p>0.05$ ).

oxytoca strain DRY14 exhibited an optimum growth at 2.0 g l<sup>-1</sup> SDS concentration (Shukor *et al.*, 2009). Literature search for other SDS-degrading isolates showed a maximal limit of between 1.0 (Margesin and Schinner, 1998; Dhouib *et al.*, 2003) and 2.0 g l<sup>-1</sup> (Chaturvedi and Kumar, 2011a; 2011b) of SDS as a substrate. Growth inhibition above this limit is probably attributable to toxicity of SDS. Fig. 5 shows at the tolerable initial concentration of 2 g l<sup>-1</sup>, approximately 90% of SDS was degraded after an incubation period of eight days where cellular growth had reached equilibrium at this point. However, there was a lag period of approximately three days before bacterial growth started to increase concomitant with reduction in SDS concentrations implying that adaptation of the bacteria to produce enzymes responsible for SDS degradation occurs within this period. 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 was attributed to microbial degradation. Margesin and Schinner (1998) reported that consortia of microbes were able to degrade 0.5 to 1 g l<sup>-1</sup> SDS in four days at 10°C. The tropical isolate *Klebsiella oxytoca* strain DRY14, isolated

**Table 1 :** Growth of strain DRY15 on detergents.

Detergent	Type	Growth
Tergitol NP9	Nonionic	+
Tergitol 15S9	Nonionic	+
Witconol 2301 (methyl oleate)	Nonionic	+
Sodium dodecylbenzene sulfonate	Anionic	+++++
Benzethonium chloride	Cationic	-
Benzalkonium chloride	Cationic	-

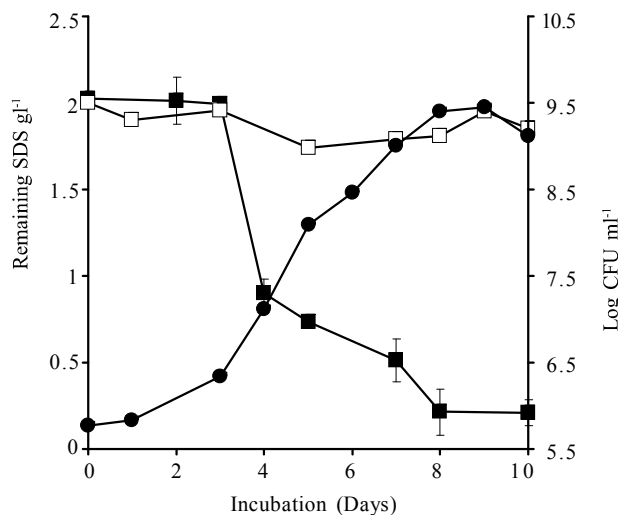


**Fig. 4 :** The effects of sodium dodecyl sulphate concentrations on the growth of strain DRY15. Data is mean standard error (n=3). Values with the same letter are not significantly different ( $p>0.05$ ).

from a detergent-polluted site, did not exhibit any lag phase during its degradation of 2 g l<sup>-1</sup> SDS, implying that the genes for detergent degradation are quickly expressed upon contact with a detergent such as SDS (Shukor *et al.*, 2009). Other SDS-degrading strains such as *Pseudomonas betelli* and *Acinetobacter johnsonii* could degrade about 0.5 g l<sup>-1</sup> SDS only after 5 days of incubation (Hosseini *et al.*, 2007). The most efficient SDS-degrading bacterium so far is a mutant form of *Pseudomonas aeruginosa* MTCC 10311 having the ability to degrade a high initial SDS concentration of 1.5 g l<sup>-1</sup> in just two days (Ambily and Jisha, 2012). The overview so far demonstrates the differential efficacy of SDS-degrading bacteria available for comparison.

**Growth on other anionic and anionic surfactants :** SDBS most often used in laundry detergent is a linear primary alkylbenzene sulfonate. The results (Table 1) showed that Tergitol NP9, Tergitol 15S9, Witconol 2301 and the anionic SDBS supported growth with the highest growth showed by SDBS. SDBS-degrading bacteria have been reported in literature (Hashim *et al.*, 1992; Perales *et al.*, 1999; Asok and Jisha, 2012) while there are very limited data on the use of Tergitol, Witconol and the cationic detergents benzethonium chloride and benzalkonium chloride as carbon source. The genus *Pseudomonas* is known for its xenobiotics-degrading capacity including detergent (Patil and Chaudhari, 2011, Dinu *et al.*, 2011; Asok and Jisha, 2012; Usharani *et al.*, 2012) and we intend to screen for other xenobiotics-degrading capacity of this bacterium with potential applications in cold-climate environment.

**Growth kinetic studies :** The correlation coefficient value for the substrate inhibition model of Haldane was 0.97



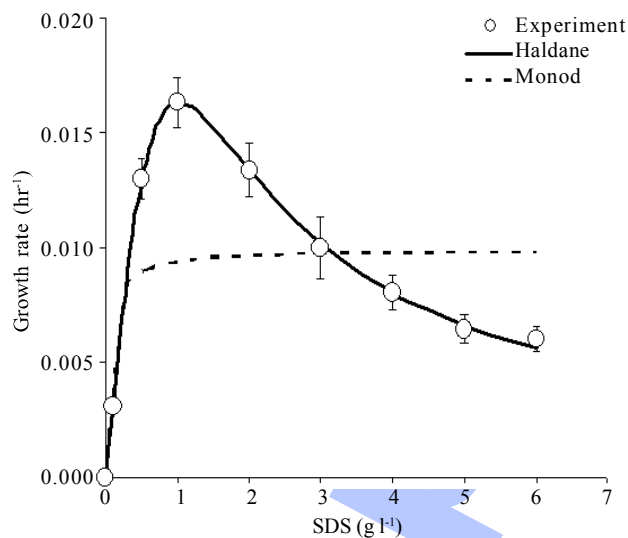
**Fig. 5 :** Sodium dodecyl sulphate degradation (■), abiotic control (□) and cellular growth (●) of strain DRY15 after growth optimizations. Data is mean ± standard error (n=3).

indicating good fitting while the Monod model gave a correlation coefficient value of 0.36 indicating poor fitting (Fig. 6). The value of specific growth rate tends to increase as the substrate concentration is increased and rises to a peak value and finally decreases. The maximum growth rate  $\mu_{max}$  was  $0.372 \text{ hr}^{-1}$  while the saturation constant or half velocity constant  $K_s$  and inhibition constant  $K_i$ , were 0.094% and 11.212 % SDS, respectively. There are few reports available on SDS-degradation and utilization kinetics. The  $\mu_{max}$  value was higher than values reported for a co-culture mixture of several SDS-degrading bacteria such as *Acinetobacter calcoaceticus*, *Klebsiella oxytoca* and *Serratia odorifera* that ranged between 0.21 and  $0.26 \text{ hr}^{-1}$  (Khleifat, 2006).

Due to the limited literature on the variety of cold-adapted SDS-degrading bacteria, this work sought to increase the repertoire of bacterial species able to degrade the anionic surfactant. In the present study, isolation of SDS-degrading bacterium from Antarctica was successfully carried out. The bacterium exhibited psychrophilic optimum growth temperature and was able to tolerate relatively high amount of SDS. In addition, the bacterium could also degrade SDBS. In future, bases in Antarctica could have collection point from wastewater with detergent-degrading microbes added in free or immobilized forms to expedite removal. Current works include purification and characterization of the alkylsulfatase from this organism, and immobilization of the bacterium.

#### Acknowledgments

This work was supported by the research grants from The Academy of Science Malaysia (ASM) and The



**Fig. 6 :** Growth kinetics of strain DRY15 on SDS. Data represents mean ± SEM, n=3.

Ministry of Science, Technology and Innovation (MOSTI). We thank Dr. Susana Claudia Vázquez and Dr. Edgardo Hernandez from The Argentinean Institute of Antarctica (IAA) for providing site support during the expedition to Antarctica.

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