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Characterization of Sodium Dodecyl Sulphate–degrading *Enterobacter cloacae* sp. STRAIN AaMa

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ABSTRACT

Surfactants are substances that can reduce its surface tension during dissolved in water or liquid and produce foam or solid. Bacterial-degrading SDS can be used for the bioremediation of this toxic substance in aquatic bodies or in soil. In this study, the isolation, identification and characterization of a local SDS-degrading bacterium is reported. Samples were isolated from a local location that had a history in surfactant contamination. Screening results shows that the best SDS-degrader was identified as *Enterobacter cloacae* sp. strain AaMa. The optimum conditions for the *Enterobacter cloacae* sp. strain AaMa to degrade the SDS were at pH 7.5, temperature 30 °C and the best nitrogen source to degrade the SDS was sodium nitrate. The $K_{m\,(app)}$ and $V_{max\,(app)}$ of SDS-degrading enzyme were 0.1035 mM and 0.4851 µmol SDS per minute per mg protein, respectively.

INTRODUCTION

Anionic surfactants is under a group of xenobiotic compounds that are made up with sulfonated or ester sulfate groups which are used commercially as ingredients in many products such as cleaning agent, detergent and also cosmetics [1–3]. In the past, surfactant contaminations of the environment followed a shift through the use involving soap-based detergent to synthetic surfactant. Due to surfactants being largely consume among consumers, they have the potential for large disposal into the water and terrestrial environments [4–6]. Because of their own favorable physicochemical properties, synthetic surfactants tend to be extensively used in many areas of technologies and research for example in pharmaceutical, cosmetics, industry of textiles, farming and also biotechnology [7]. Surfactant output is expected to increase by 18% by 2022, from its current level of 14.1 million tonnes in 2017 to 12.5 million tonnes in 2006 and 14.1 million tonnes in 2017 [8]. Although surfactants are basically non-toxic to human, there is certainly a common level which their presence in drinking water is undesirable. Due to the latest laws on banning the important of microorganisms along with the extra care of the use of genetically modified organism (GMO) to be used for the purpose of bioremediation regarding xenobiotics in Malaysia, it is very important to isolate the local bacteria for bioremediation involving anionic surfactants in Malaysia [9–11]. The highly concentrated amount of SDS in the environments is a serious problem that can cause the alteration of hormonal system and also biological functions in the balancing the ecosystem. It is also stated that SDS is toxic to human health and other aquatic life [12].

The degradation of the SDS well help to ensure the low concentration of SDS in the environment. Bioremediations is the most potential technique to treat contaminated environment. The studies of degradation of pollutants into the less dangerous state using the ability of the microorganism can be the key to save nature. Probably the most extensively studied anionic surfactants are alkylsulphates (AS) as well as linear alkylbenzene sulphonate (LAS). Microorganisms harbors the alkylsulphatase enzymes which separate SO_4^{2-} from alkylsulphate esters. Report demonstrated that a few microorganisms which are capable degrading surfactant pollutants within the environment have already been identified. A few examples are shown in **Table** 1.

Table 1. List of microorganisms that can degrade SDS.

Microorganisms	References
Pseudomonas koreensis YRW-02	[13]
Pseudomonas songnenensis YRW-05	[13]
Pseudomona sp.	[14]
Paraburkholderia tropica	[15]
Pseudomonas sp. strain Maninjau1	[15]
Pseudomonas helmanticensis	[16]
Bacillus amyloliquefaciens strain KIK-12	[17]
Pseudomonas sp.	[18]
Enterobacter sp strain Neni-13	[10]
Delftia acidovorans	[19]
Pseudomonas putida	[12]
Pseudomonas alcagenes	[12]
Klebsiella oxytaca	[20]
Citrobacter braaktii	[21]
Pseudomonas C12B	[22]

SDS or Sodium Dodecyl Sulphate is a primary alkyl sulphate. The pathway started by sulphatase- hydrolysis of ester to an alcohol and an inorganic substance. SDS was study using ^{14}C-radiotracer in order to get the degradation pathway by *Pseudomonas* sp. C12B that been found a component of dodecanol and a trace of dodecanal. Then it was oxidized into fatty acid dodecanoic which than elongated to tetradecanoic acid. These fatty acids are incorporated as the acyl chains in membrane phospholipids and also formed a water soluble product that is tricarboxylic acid (TCA) cycle [23]. The aim of this study is to screen for SDS-degrading bacteria from local soil samples to isolate potential SDS-degraders that can be used to remediate SDS-polluted water bodies or soils in a Malaysian scenario.

MATERIAL AND METHODS

Isolation of SDS degrading Bacteria

Bacterial Sampling

Sample was collected from a location that had some history from soil contaminated with detergents. This site was chosen as it is used to be polluted with animal waste and heavily polluted with cleaning agent. The location of the sampling was at the vicinity of the Biotech 2 building in June 2016.

Isolation of bacteria

Bacteria were isolated from soil that was contaminated with detergent by a technique of enrichment in basal salt medium (BSM) that contained sodium dodecyl sulphate (SDS) as a carbon source. 5 g of solid samples were inoculated in 45 mL sterile SDS basal salt media in 250 mL conical flask. The isolates where incubated at room temperature (25 °C) on orbital shaker at 150 rpm for 3 days. SDS basal salt medium contained (gL^{-1}): KH_2PO_4, 1.36; Na_2HPO_4, 1.388; KNO_3, 0.5; $MgSO_4$, 0.01; $CaCl_2$, 0.01; $(NH_4)_2SO_4$, 7.7. The medium also contained the following trace elements containing (gL^{-1}):): $ZnSO_4.7H_2O$, 0.01; $MnCl_2.4H_2O$, 0.01; H_3BO_4, 0.01; $CoCl_2.6H_2O$, 0.01; $FeSO_4.2H_2O$, 0.01; $CuCl_2.2H_2O$, 0.01; $Na_2MoO_4.2H_2O$, 0.01.

The medium was supplemented with filter sterilized SDS as a carbon source with a final concentration of 1.0 g/L [21]. For preparation of SDS solid media, 20 g/L of bacteriological agar was added. After 3 days of incubation, 5 mL of culture was transferred to 45 mL of fresh BSM in 250 mL conical flask. After five serial subcultures, a 1-mL sample was taken from the final enrichment flask, serially diluted (10^{-5}), and plated on nutrient agar (NA) plate. The plate was incubated until there are some formations of bacteria. The colonies were chosen based on the differentiation of size, shape, edge, elevation, surface and also colour. A single colony was picked and grown in Nutrient broth (NB). The subculturing process was done every 2 weeks in order to get fresh culture for any experiment purposes. The four selected isolated from preliminary screening were chosen for secondary screening where the secondary screening is to test the ability of the bacteria to degrade higher concentration of SDS. 100 µL of each isolate was pipette into BSM that containing 1g/L SDS as a carbon source. In order to determine the degradation of the SDS it was measured daily by using MBAS assay [24].

Methylene blue active substances (MBAS) assay

Acidic Methylene blue reagent (3.13 mM; pH 5-6): 0.1 g of methylene blue was prepared by dissolving in 100 mL of 10 mM tetraborate buffer solution. Then the pH was adjusted between 5-6 and kept in topaz-colored flask. Sodium tetraborate buffer (50mM; pH 10.5) was prepared by dissolving 19 g of sodium tetraborate decahydrade in 850 mL of distilled water in a volumetric flask and the pH was adjusted until 10.5 and was topped up until 1 L with distilled water.

Samples of the isolates were diluted with distilled water to the final concentration of SDS of 2.0 mg/L in a final volume of each sample to be 5 mL. 200 µL of 50mM sodium tetraborate pH 10.5 was added in the dilution samples followed with 100 µL acidic methylene blue reagent. Then the mixture was mixed using a vortex. Then, 4 mL of chloroform was added in the mixture and stirred for 30 seconds, and the mixture let to be separated for 5 minutes. The process was conducted using a separating funnel in order to get the chloroform layer. Next, the layer of the chloroform was read using a spectrophotometer at the absorbance of 650 nm using a glass cuvette [24].

Identification

For further studies, the isolate that shows the ability to degrade 1 g/L of SDS was further identified by observing the morphology on NA plate, microscopic observation and also through 16S rDNA molecular phylogenetic analysis.

16S rRNA analysis

To obtain a more precise identification, the 16S rRNA analysis was carried out. This identification invlves a genomic DNA extraction of bacteria, the polymerase chain reaction (PCR), sequence analysis and also the identification of the sequence. The genomic DNA extraction of selected isolates was extracted using GF-1 Nucleic Acid Extraction Kit by Vivantis Technology according to the manufacturer's instruction given. The DNA that had been harvested at the end of the protocols was used as a template for PCR. The PCR was carried out using the 16S universal primer as before [20].

The PCR was done by an initial denaturation at 94 °C at 4sec, denaturation for 94°C for 30sec, annealing for 56 °C for 30 sec, extension for 2 steps of 34 cycles of 72°C for 4 sec and the final extension 72°C for 4min. The detection of unpurified PCR product was done by electrophoresis using 1% of agarose gel. The electrophoresis was carried out for 90 min at 60 V. The PCR product was sent to Genomics BioScience and Technology Co., Malaysia, for sequencing. Then the bases were compared using BLAST available at the NCBI website. The 16s rRNA sequence was submitted to GenBank under the accession number **MF102229**.

Phylogenetic tree analysis

Twenty closest 16s rRNA sequences from the BLAST website were subjected to multiple alignment from the data that closely matched to the selected isolate were chosen and then aligned using Clustal_W. The phylogenetic tree was built by using Mega7 with *Rhodococcus qingshenqii* strain djl-6 as an outgroup in the cladogram.

The growth optimization of the bacterial
The optimization condition was done in order to find the best condition for the isolate to degrade the SDS. The growth condition of isolated were measured based on the pH (5.5 - 10.0), temperature (20- 40 °C), and also the nitrogen sources (Sodium nitrate, Ammonium sulphate, glycine and ammonium chloride). The degradation of bacteria was measured using MBAS assay.

Optimization of pH
The optimization of the pH was carried out by inoculating the single colony of isolate for 24h into 10 mL BSM that contain 1 gL^{-1} of SDS containing a phosphate buffer at different pH (5.5 – 10.0) in triplicates and incubated for incubated for 3 days on orbital shaker with a constant speed of 130 rpm. The degradation of SDS was measured using MBAS assay.

Optimization of nitrogen sources
This experiment was done to find the best nitrogen sources for the degradation of SDS by the selected isolate. 4 commonly use nitrogen sources were chosen in this study which was sodium nitrate, ammonium sulphate, glycine and ammonium chloride. 100 µL of 24 h culture was grown in the BSM containing 1 gL^{-1} of SDS and incubated for 3 days on orbital shaker with a constant speed of 130 rpm. The degradation of SDS was measured using MBAS assay.

Optimization of temperature
The optimization of the temperature was done by incubating the isolate in different incubation temperature. 100 µL of 24 h culture was grown in the BSM containing 1 gL^{-1} of SDS and incubated for 3 days at different temperature (20- 40 °C) on orbital shaker with a constant speed of 130 rpm. The degradation of SDS was measured using MBAS assay.

Preparation of enzyme extracts
On day 2, bacterial cells were harvested by centrifuging a 1 L culture at 10,000 x g for 10 minutes at 4 °C. Then the cell pellets were resuspended in 50 mM Tris-HCl at pH 7.5 it also contain the mixture of 1 mM PMSF and 2 mM of DTT. For 30 minutes, the cells were sonicated with intermittent cooling Then, the crude extract was centrifuged at 10,000 ×g for 20min. The supernatant was ultracentrifuged at 105,000 ×g for 2 h. The supernatant that containing high SDS-degrading activity was later on be subjected to enzyme assay. Protein content was determined by the Bradford method or also known as Coomassie blue protein assay The calibration was done with the concentration of bovine serum albumin (BSA) ranging from 0 to 20 µg/mL. 20 µL of protein and 200 µL of Bradford reagent were pipetted into a 96-well microtiter plate. Then the mixture was incubated for 5 min before the microtiter plate was read at 595 nm.

Alkylsulfatase assay
The alkylsulfatase activity in cell was obtained by incubating the 50 µL of enzyme of the isolate with 450 µL 50 mM Tris-HCl and add on 500 µL (0.1-10.0 mM) SDS. To stop the reaction, the 100 µL of 20% trichloroacetic acid (TCA) was added. The loss of substrate was measured by the MBAS assay as described before [24].

RESULTS AND DISCUSSION

Isolation of SDS-degrading bacteria
Four bacterial isolates that have the capability to survive in SDS BSM were isolated from soil samples. The screening of SDS-degrading bacteria was carried out in order to find out the best isolate that was able to degrade the SDS. The degradation of SDS was measured daily using the MBAS assay. From the study, isolate AaMa provide the highest degradation which was 96% of SDS being degraded for 3 days of incubation compared to isolate B, C and D (**Fig. 1**). Isolate B shows the lowest degradation which was only 53% of SDS was degraded after day 3 of incubation. The control that was not using any inoculated medium shown no degradation during the 3 days of incubation which means that the SDS degradation did not happen.

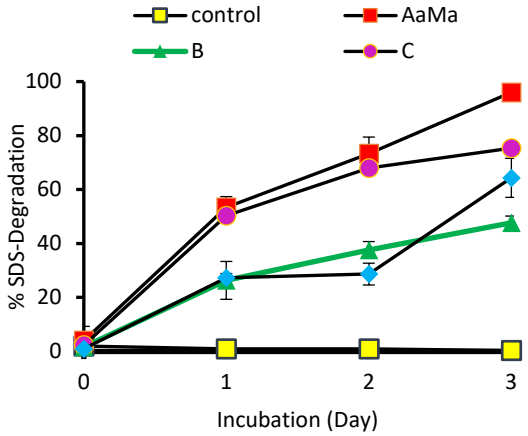

Fig. 1. Four most potent isolates that are capable to degrade SDS that was grown in BSM containing 1 g/L of SDS.

Identification of SDS-degrading bacteria

Morphological observation
Based on the screening, isolate AaMa was chosen as the best SDS-degrading bacterium. Isolate AaMa was streaked on the nutrient agar to study on the morphology of the isolates. Nutrient agar is a general nutrient rich media that is commonly used to grow a vast range of bacteria. Several elements in the nutrient agar such as peptone and beef extract supply the amino acids, minerals, and other nutrients used by varied microbes for their growth The morphology characteristics of the isolates were circular, the surface is rough, and the color of the colonies was yellow (**Fig. 2**).

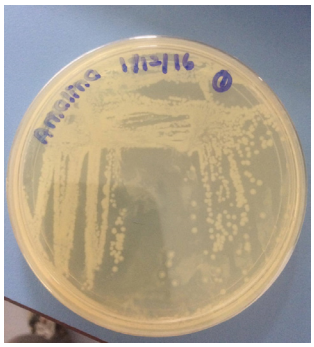

Fig. 2. Colonies form of isolates AaMa using a streaking technique after incubation for 24 hours at room temperature.

A single colony was then inoculated into BS media supplied with SDS at concentration of 1.0 g/L. The SDS acts as the sole carbon and energy sources. After 3 days incubation, the presence of single colonies in the SDS agar plate can be seen. The culture was then inoculated into 10 mL of sterile nutrient broth and incubated for 24 hours at the incubator shaker. **Fig. 3** shows

bacteria growth of Isolate AaMa in BSM containing SDS after 3 days grown at room temperature.

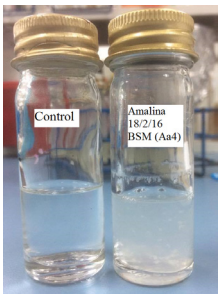

Fig. 3. Bacteria growth of Isolate AaMa in BSM containing SDS after 3 days grown at room temperature.

Biochemical test

Gram staining

Gram positive or Gram-negative differentiation is based on the properties on the cell wall. In this study, isolate AaMa was identified as a Gram-negative bacterium. As shown in **Fig.** 4, the bacterium shown a rod shaped. In another study, a surfactant-degrading *Enterobacter* sp. is isolated from a soil contaminated with surfactant [10,25,26]. Other surfactant-degrading bacteria that have been reported are *Pseudomonas* sp. strain C12B [23], *Comamonas terrigena* [27], *Citrobacter braakii* [21], *Bacillus cereus* [28] and other bacterial genus such as *Acinetobacter, Pantoea, Vibrio, Flavobacterium, Pseudomonas, Enterobacter, Actinomyces, Eschericia, Shigella, Proteus, Anaebena, Corynebacterium and Staphylococcus* (**Table 1**).

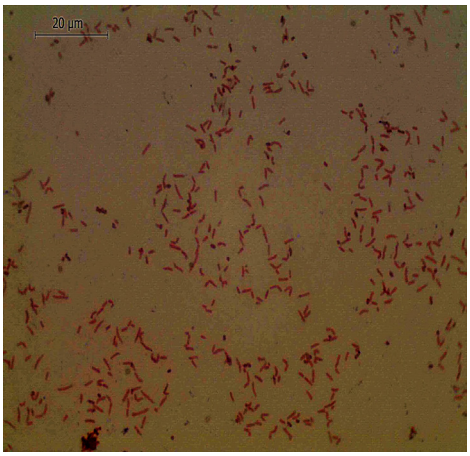

Fig. 4. Cellular morphology of isolate AaMa under the light microscope with the magnification of 1000x using Olympus Trinocular BX40.F4.

16S rRNA analysis.

16S rRNA analysis showed that this isolate AaMa was similar to *Enterobacter cloacae* sp. Previous studies have also shown that bacteria from the Enterobacter family are good SDS degraders (**Table 1**). *Acinetobacter calcoaceticus* and *Pantoea agglomerans* [29], *Citrobacter braakii* [21] and *Comamonas terrigena* [27]. The sequences were aligned at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The analysis from GenBank database showed isolate AaMa sequences was closely related to *Enterobacter cloacae* strain ATCC 23373 with maximum align of 98%. **Fig. 5** shows a neighbor-joining phylogenetic tree. *Rhodococcus qingshenqii* was used as the outgroup in thecalodgram.

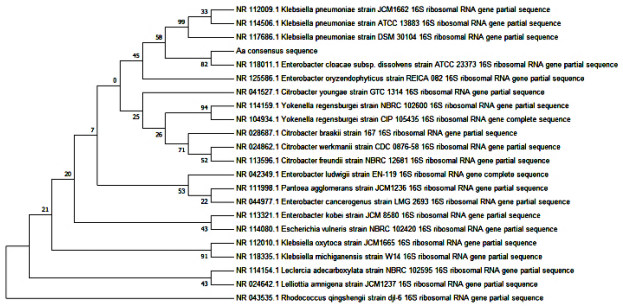

Fig. 5. Neighbour-joining method cladogram showing phylogenetic relationship between isolate AaMa and other related reference microorganisms based on 16S rRNA gene sequence analysis. *Rhodococcus qingshenqii* strain djl-6 is the outgroup.

Optimization of isolate AaMa

Different parameters were chosen for optimization to increase its ability to degrade the SDS. The parameters tested were including the optimization of pH, temperature and nitrogen sources. All results were analyzed statistically.

Optimization of pH

Optimization of pH provides the importance to the growth of the bacteria since pH will strongly affect the bacterial growth and also importance bioremediation experiment. The effect of the pH on the degradation of SDS by isolate AaMa was studied using phosphate buffer with pH range of 5.5 until 10.5 (**Fig. 6**). From this study, the optimum pH for degradation of SDS was at the pH 7.5 with 100% of SDS elimination. Most bacterial functioning really well at pH neutral to slightly alkaline in range between pH 5 until pH 9 with the most optimum condition would be pH 7 [30]. The degradation decreased mostly at the pH 10.5 with only 8% of SDS elimination. The previous studies shown the different pH for different bacteria, but still in the range pH 5 to 9 in most SDS-degrading bacteria [9,12–17,19,21,25,31–44].

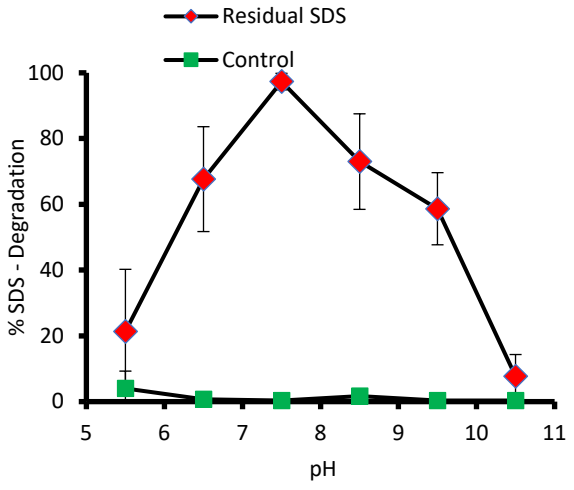

Fig. 6. The effect of pH on the degradation of SDS by *Enterobacter cloacae* sp. strain AaMa with SDS concentration 1g/L.

Optimization of temperature

Different temperatures on the SDS biodegradation in BSM containing 1 g/L of SDS carried out at the temperatures ranging from 20 to 40°C (**Fig. 7**). Temperature is one of the most important factors that may influence the biodegradation. At low temperature, the metabolic rate will fall below the satisfactory

level while the high temperature will inactive the enzyme in microorganism and also the lipid bilayer of plasma membrane melts and cell could disintegrate [45].

Based on this study, the degradation of SDS was increased when the temperature was increased until it reached the optimum temperature which was 30°C. After the optimum temperature, the degradation of the SDS decreased slowly. From previous studies, the result showed that it was still in the range of optimum temperature seen in most SDS-degrading bacteria of between 25 and 37 °C [9,12–17,19,21,25,31–44], with the exception of a few psychrotolerant bacteria [46].

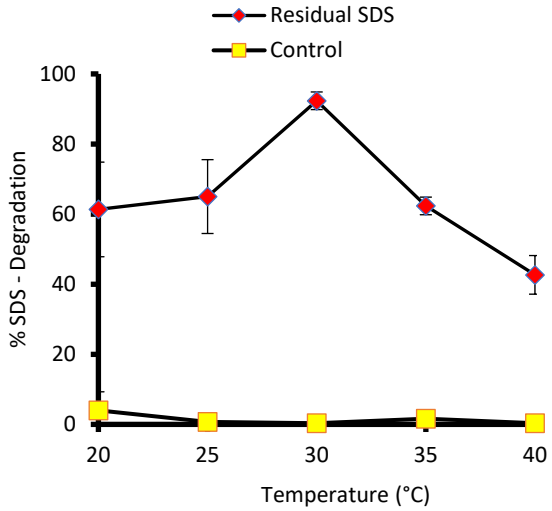

Fig. 7. The effect of temperature on the degradation of degradation of SDS by *Enterobacter cloacae* sp. strain AaMa with SDS concentration 1g/L.

Optimization of nitrogen sources

Nitrogen sources also play the important role in the biodegradation. Nitrogen plays an important role as an element in bacteria cell, provide a structural and functional to cells of the bacteria. The results of the study demonstrated how various nitrogen sources affect SDS breakdown (**Fig. 8**). The results showed that the optimum source of nitrogen for the isolates AaMa to degrade SDS was sodium nitrate. But from the previous studies, the best nitrogen source that commonly reported to be best nitrogen source was ammonium sulphate such as *Citrobactor braakii* [21], *Comamonas terrigena* [27] and in nearly all sDS-degrading bacteria [9,12–17,19,21,25,31–44]. To aid bioremediation solutions for surfactant pollution, determining the best nitrogen supply is essential [30].

Enzymatic Activitiy of Alkylsulfatase

The effect of the concentration of substrate on alkylsulfatase activity was then studied. The alkylsulfatase enzyme produced the normal Michaelis-Menten curve (**Fig. 9**) when responded to SDS. The curve that was generated by the Graphpad Prism™ software gave values of $K_{m\,(app)}$ and $V_{max\,(app)}$ of 0.1035 mM SDS and 0.4851 µmol/min/mg protein respectively. Correlation coefficient of 0.94 shows reliability of the regressed data. The K_m and V_{max} of the alkylsulfatase from the bacterium *Pseudomonas putida* FLA using the Lineweaver-Burke plot were found to be 1.0 mM and 10.9 µmol SDS per minute, respectively [47].

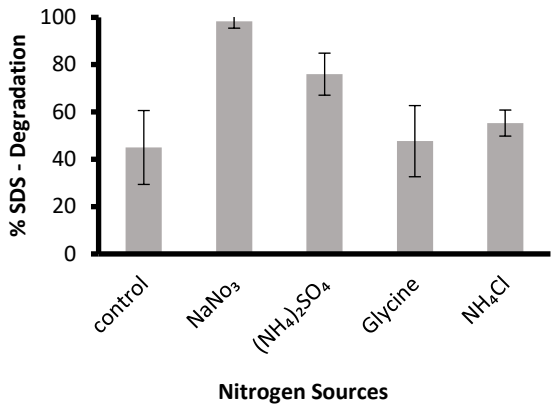

Fig. 8. The effect of 0.1% different nitrogen sources on the degradation of SDS by *Enterobacter cloacae* sp. strain AaMa with SDS concentration 1g/L.

A lower K_m value of 34 µM was demonstrated by *Pseudomonas putida* S-313 [48]. The low value of $K_{m\,(app)}$ showed high affinity towards substrate. $V_{max\,(app)}$ value was lower compared to previous study. Substrate concentrations, activators, enzyme-specific inhibitors, and non-specific actions of substances like salts and buffers are all candidates for influencing the value. The value might be impacted by factors such as the optimal pH and temperature during the enzyme assay, as well as by interactions with other proteins or membranous material that may be present [48].

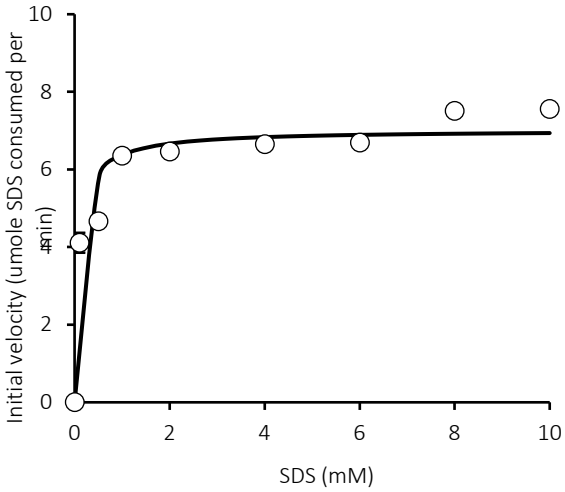

Fig. 9. Michaelis-Menten curve for alkylsulfatase substrate with SDS as the. Assays were carried out at room temperature and at pH 7.5.

CONCLUSION

A locally isolated potent SDS-degraded bacterial *Enterobacter cloacae* sp. strain AaMa was successfully isolated, characterized and being identified. The optimization of the various conditions to obtain the best degradation was successfully done. The optimum conditions for the growth and the degradation of SDS of this isolate were at the pH 7.5, temperature 30 °C and the sodium nitrate was found the best nitrogen source for the degradation. The optimization using different parameters help to increase the degradation of SDS by selected isolate.

The species of the isolate was identified using 16S rRNA analysis as *Enterobacter cloacae*. The $K_{m\ (app)}$ and $V_{max\ (app)}$ of SDS-degrading enzyme are 0.1035 mM and 0.4851µmol SDS per minute per mg protein respectively. The ability of this organism to utilize detergent could be of importance on bioremediation technologies in Malaysia. However, more studies need to be conducted such as studying the effect of heavy metals to SDS degradation, the effect of immobilization of the bacterium and to further purify and characterize the enzyme produced in this study. The isolate has to be determined not to have any undesirable effects on the environment and public health.

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