ADVANCES IN BIOSCIENCES

VOLUME 1

Enzymes are macromolecular biological catalysts that is important to speed up reaction. Enzymes can be found naturally from the microorganisms such as bacteria, fungi and yeast which may not be genetically modified. In this perspective, resorting to bioremediation to clean-up such environment may prove feasible and beneficial as microorganisms effective in degrading such substances. The liberation of excess halogenated compounds into the environment is becoming a major global issue, since the toxic contaminants tend to accumulate and persist in the biosphere. Some of the microbes that able to utilise these toxic compounds have been successfully isolated from the soil contaminated environment and were further characterised. Apart from pollutant degradation, commercial enzyme like protease will be discussed that has potential for commercialization in many manufacturing processes.

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Editors

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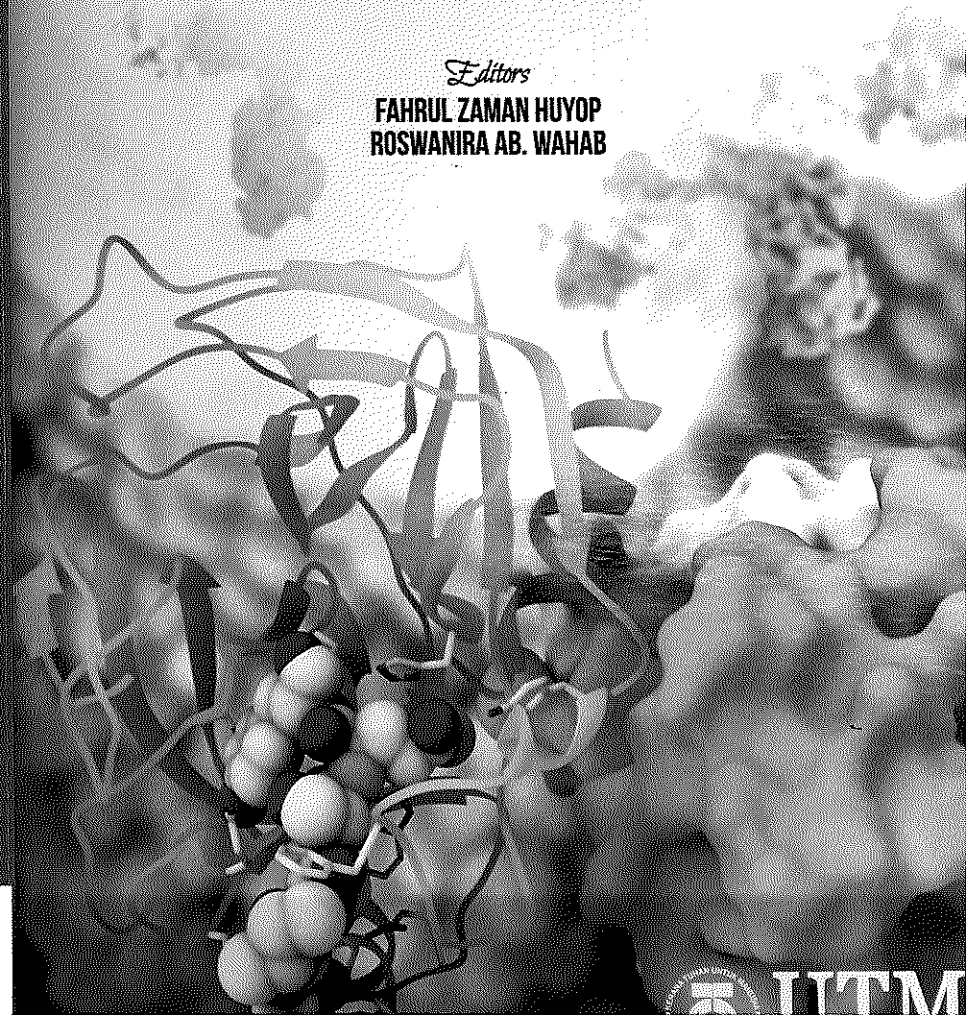

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Identification of an Unknown Bacteria and Evaluation of Its Potential Use in Bioremediation

Hassana Abubakar, Mohamed Faraj Edbeib and
Azzmer Azzar Abdul Hamid

5.1 INTRODUCTION

The most common environmental contaminants are halogenated organic compounds which are released into the biosphere through their use in agricultural, chemical and pharmaceutical industries. These compounds are broadly found in the environment either naturally or as a consequence of pesticides and herbicides application (Alomar *et al.*, 2014). They are not easily degraded, thus are hard to be removed from the environment. The accumulation of these compounds is causing severe pollution and human health problems such as endocrine disruption, genotoxicity, as well as detrimental consequence on immune and reproductive systems because they are toxic and persistent (Hertz-Picciotto *et al.*, 2008).

Fortunately, various microorganisms can benefit from these toxic compounds by using them as carbon source as well as an electron donor. Microbial degradation of halogenated compounds has been investigated since then and until now a number of microorganisms having dehalogenases have been isolated. Microbial degradation significantly contributes to the lowering of halogenated compounds accumulation in water systems and soil.

This biological method is economical, safer and environmentally friendly, hence give a suitable choice to dispose these environmental contaminants. Considering the negative effect to man and the environment associated with the abundant deposition of these halogenated compounds, there is need to isolate and identify more species of microorganisms that can degrade these compound to aid in their removal from the environment. However, various species of microorganisms that can degrade halogenated compounds from contaminated soil have been documented but very few from contaminated water.

5.2 GROWTH CONDITION OF BACTERIA SA1

Bacteria SA1 previously isolated from contaminated waste water was successfully sub-cultured onto nutrient agar (NA) at 30°C for 24 hours. The growth of the bacterial strain was observed on nutrient agar plate (Figure 5.1). The bacterial culture was then inoculated into three flasks containing, 10 mM, 20 mM and 30 mM 2,2-dichloropropionic acid (2,2-DCP) as the sole source of carbon and energy. After 24 hours of incubation, 0.1 mL of aliquot from each flask were spread onto their respective solid minimal media containing 10 mM, 20 mM and 30 mM 2,2-DCP as a carbon source. After 5 to 8 days of incubation, the colonies formed was further characterised as reported by Abel *et al.* (2012).

5.3 GRAM- AND SPORE STAINING

The Gram-staining and spore staining were observed under light microscope at 100X magnification using oil immersion (Figure 5.2). Table 5.1 summarised all observations. Spore staining indicated that strain SA1 has no spore. Spore forming bacteria like *Clostridium*and *Bacillus* possess a green endospore enclosed in a pink sporangium (Cowan *et al.*, 2004). However, bacteria SA1 when observed under microscope showed only pink colour, no

green colour was seen as reported in previous literature (Figure 5.2 b.).

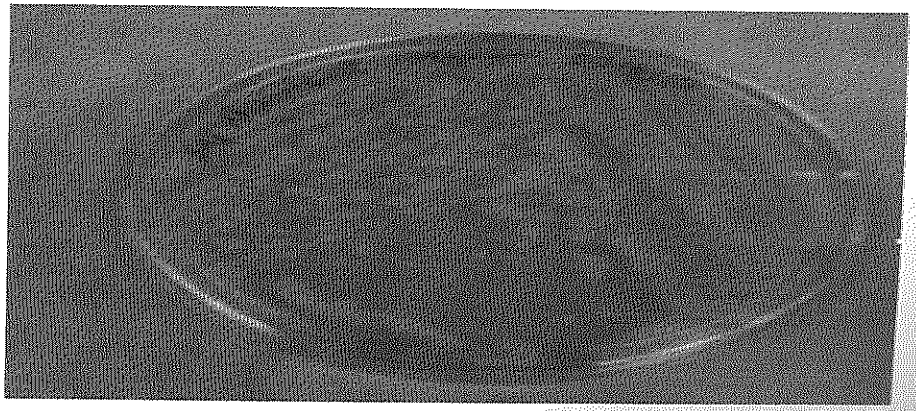
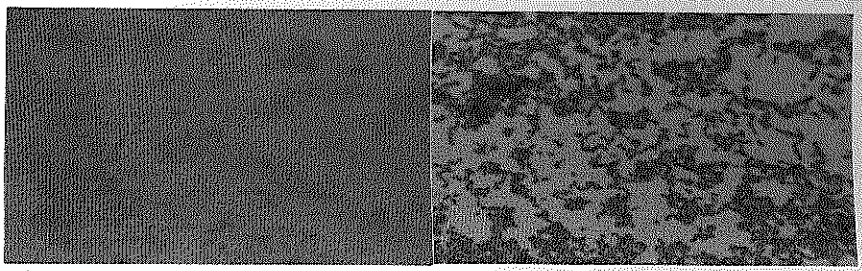


Figure 5.1 Bacteria SA1 on nutrient agar media growth at 30°C after 16 hours incubation



a) b)

Figure 5.2 Bacteria SA1 (a) Gram staining (b) spore staining

Table 5.1 Strain SA1 characterisation

Characteristic	Observation
Gram	Negative
colour	Pink
shape	Rod
spore	Negative

5.4 BIOCHEMICAL TESTS

Biochemical tests were carried out on bacteria SA1 to identify the genus of the species of the isolated bacteria. The biochemical tests carried out were motility, catalase, oxidase, citrate, urease, MacConkey agar, starch hydrolysis, gelatin liquefaction and nitrate reduction. Motility test suggested SA1 was non-motile.

The presence of catalase enzyme in bacteria SA1 was confirmed after the addition of hydrogen peroxide to the bacteria. Bubbles developed as a result of oxygen gas evolution from hydrogen peroxide decomposition by the catalase enzyme. Figure 5.3 shows the result of bacteria SA1 catalase test.

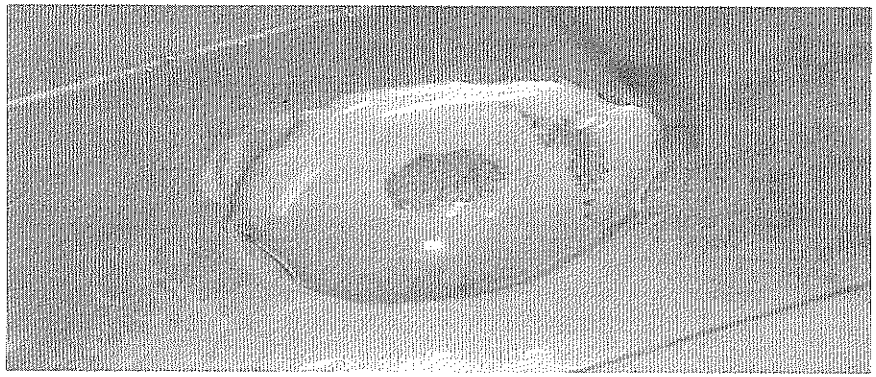
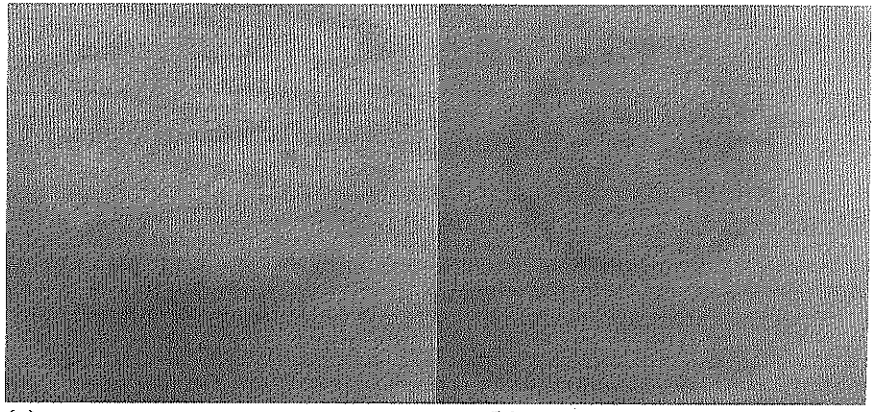


Figure 5.3 Bacteria SA1 catalase test

Bacteria SA1 oxidase test resulted no colour changed (Figure 5.4) suggesting strain SA1 is oxidase negative. Cytochrome c oxidase producing organisms are commonly identified by this test. This enzyme is involved in the transfer of electrons to oxygen in electron transport chain. Citrate test was also carried out and the result was observed as positive after incubating the sample for three days. The green citrate agar on which the bacteria was streaked changed to blue whereas the control agar remain unchanged (Figure 5.5).



(a) (b)

Figure 5.4 Oxidase test (a) bacteria SA1, (b) control

The blue colour observed in the test medium indicates that the bacteria was able to utilize the citrate as sole carbon source and energy

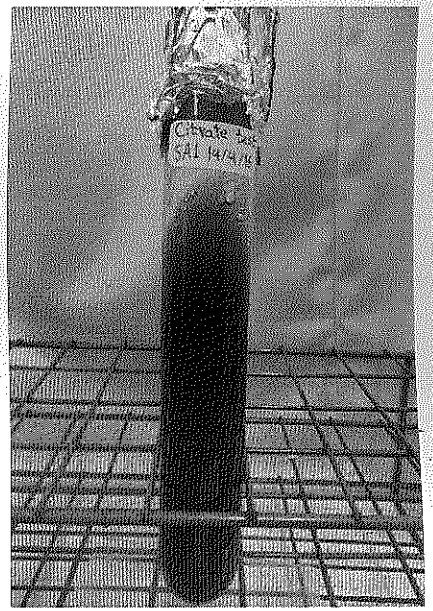
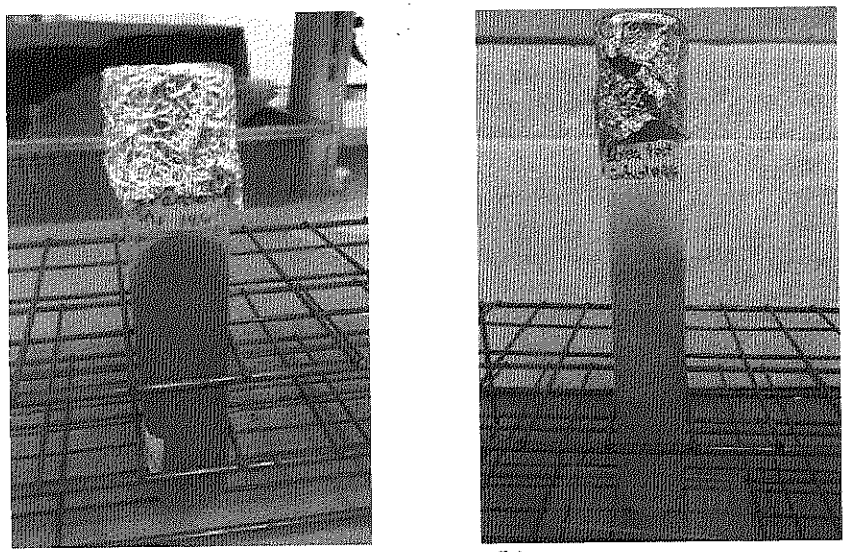


Figure 5.5 Citrate test on bacteria SA1

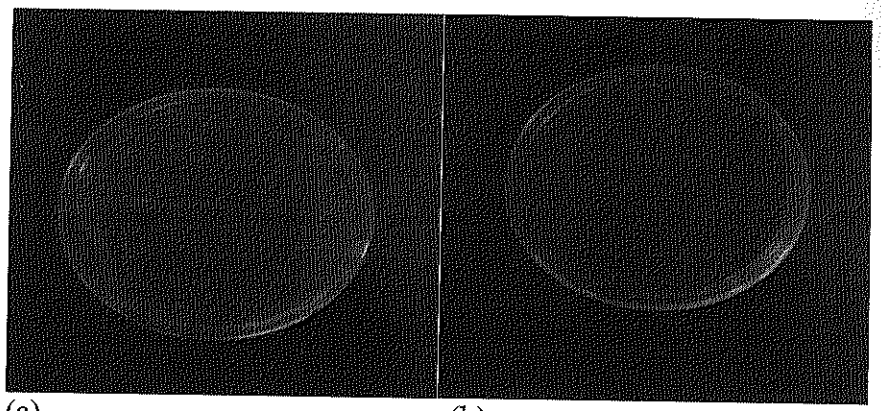
Urease test for strain SA1 was shown in Figure 5.6. The urea agar on which the bacteria was streaked changed to pink whereas the control agar remain unchanged. Pink colour indicated that bacteria SA1was able to degrade urea in the medium and produced ammonia creating an alkaline environment. This suggests strain SA1 was able to produce urease exoenzyme which hydrolysed urea to NH_3 and CO_2.



(a) (b)

Figure 5.6 Urease test (a) bacteria SA1, (b) negative control

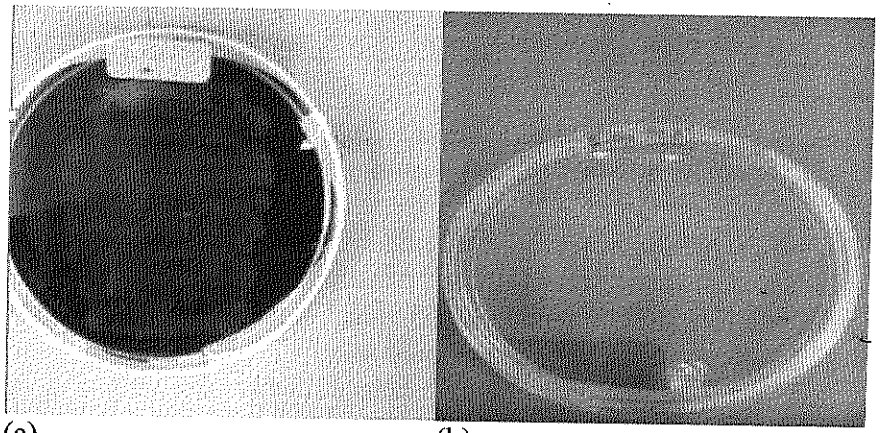
MacConkey Agar Test was positive when there was growth of the strain SA1 on the agar plate, whereas there was no growth on the control plate (Figure 5.7). The growth suggested that the bacteria was able to ferment lactose and use it for energy production. MacConkey Agar contained crystal violet and bile salts which prevents the growth of Gram positive bacteria. Strain SA1 being a Gram negative and lactose fermenting bacteria was capable of growing and forming red colonies on the agar.



(a) (b)

Figure 5.7 MacConkey Agar test (a) bacteria SA1, (b) negative control

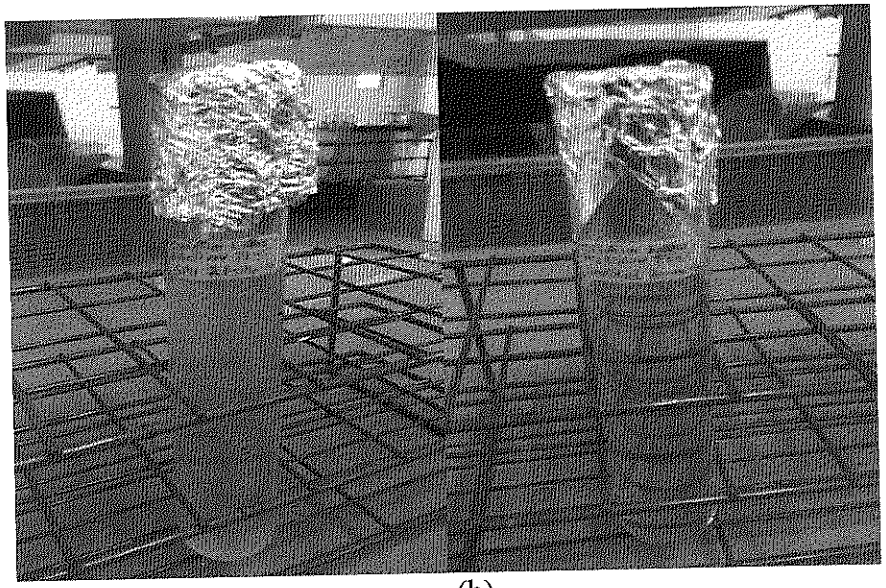
Starch hydrolysis test of bacteria SA1 was positive as shown in Figure 5.8. Red colour was observed near the growth zone after the addition of iodine which indicates that the bacteria was able to produce some exoenzymes like α-amylase and oligo-1,6-glucosidase which hydrolysed the starch in the medium.



(a) (b)

Figure 5.8 Starch hydrolysis test (a) bacteria SA1 (b) negative control

The result of gelatin liquefaction test was shown in Figure 5.9. After incubating for 6 days, the medium appeared cloudy and liquefied even when refrigerated while the control remained solid which indicates that the bacteria was able to produce gelatinase, a proteolytic enzyme which breakdown gelatin into smaller molecules (peptides and amino acids).

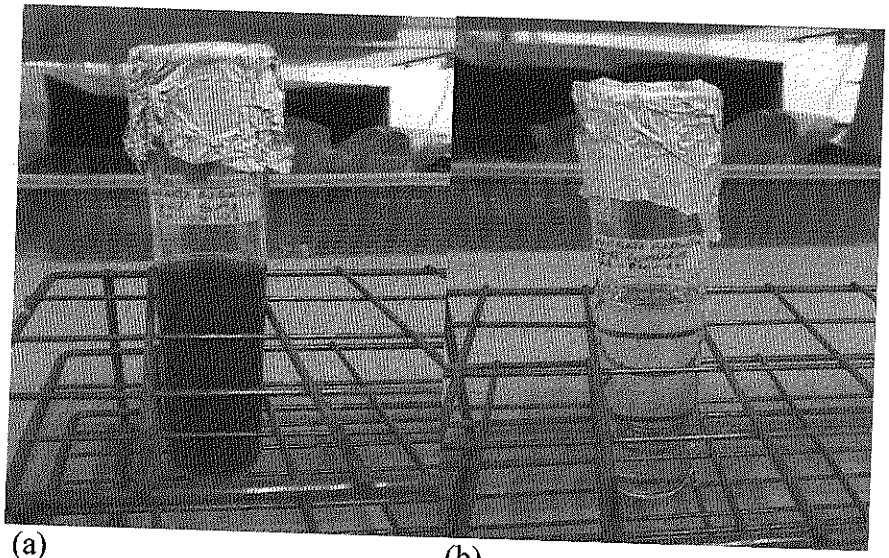


(a) (b)

Figure 5.9 Gelatin liquefaction test (a) inoculated by strain SA1, (b) control (without any bacteria)

The nitrate reduction test showed negative result (Figure 5.10). No colour was changed when10 drops of sulphanilic acid solution and naphthylamine solution were added after 5 days of incubation with the bacteria in nitrate broth incubated at 30°C. When zinc dust was added for final observation, red colour developed within 5-10 minutes which confirmed the result to be negative. The development of the red colour was due to the presence of nitrate in the medium. Nitrate was not reduced by the bacteria in the first place but later reduced by zinc to nitrite. The nitrite formed nitrous

acid, reacted with sulphanilic acid which then reacted with the naphthylamine to form the red complex. The results were summarized in Table 5.2.



(a) (b)

Figure 5.10 Nitrate reduction test (a) solution contained bacteria SA1, (b) control (without any bacteria)

Table 5.2 Staining and biochemical tests results

Tests	Results
Gram staining	Negative
Spore staining	Negative
Motility test	Negative
Catalase test	Positive
Oxidase test	Negative
Citrate test	Positive
Urease test	Positive
MacConkey agar test	Positive
Starch test	Positive
Gelation liquefaction test	Positive
Nitrate reduction test	Negative

5.5 16S rRNA GENOMIC ANALYSIS

Promega® DNA Isolation kit was used to extract the genomic DNA from strain SA1. The isolated genomic DNA was run on DNA gel to check the presence of DNA. The DNA was purified from gel using Promega® purification kit and was used as a DNA template in PCR reaction using universal primers forward (27F) 5'-AGA GTT TGG ATC MTG GCT CAG - 3' and reverse (1492R) 5'- CGG TTA CCT TGT TAC GAC TT -3' (Jiang *et al.*, 2006). The amplified 16S rRNA gene was seen at 1.5 kb in size. The PCR product was sent for DNA sequencing at First Base Laboratories Sdn. Bhd. The partial sequence of SA1 16S rRNA gene was BLASTn in NCBI database to ascertain its identity. BLASTn result showed SA1 strain has 98% sequence similarity with *Acinetobacter sp.* Ue2-1.1 gene, *Acinetobacter sp.* Y1 and other *Acinetobacter* strains (Figure 5.11).

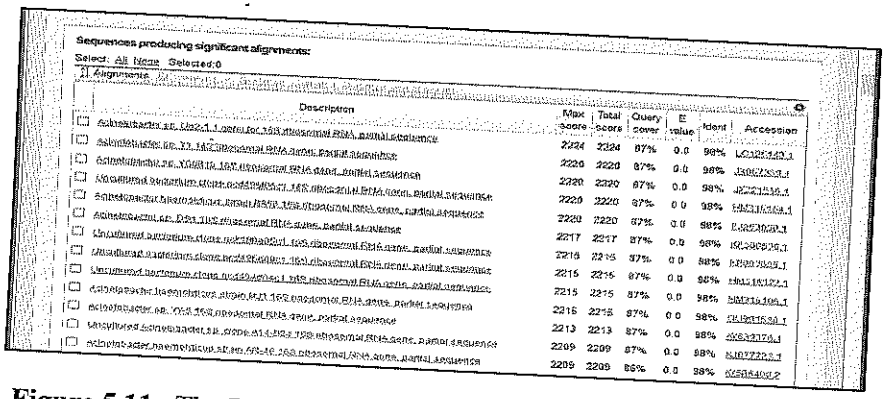


Figure 5.11 The BLASTn outcome displaying the similarity between SA1 and other species

5.6 CHARACTERISTICS OF STRAIN SA1

The staining and biochemical tests were compared to the properties of the established *Acinetobacter*strain (Table 5.3). The overall characteristics of strain SA1 matched to the *Acinetobacter baumannii* isolated by Abel *et al.* (2012) which capable of utilizing 2,2-DCP as a carbon and energy source.

Table 5.3 Comparison between SA1 and *Acinetobacter baumannii* (Abel *et al.* (2012)

Tests	SA1	*A.baumanni*
Gram staining	Negative	Negative
Motility test	Negative	Negative
Catalase test	Positive	Positive
Oxidase test	Negative	Negative
Citrate test	Positive	Positive
Nitrate reduction test	Positive	Positive

In addition, *Acinetobacter* are said to be Gram-negative bacilli, non-spore forming, non-motile, non-fermentative and strictly aerobic fit the criteria of strain SA1. All strains of *Acinetobacter species* are found to grow between 20° to 30°C but most strains have 33° to 35°C as optimal temperature. They are oxidase negative, mostly nitrate negative (Constantiniu *et al.*, 2004) and partially ferment lactose when cultured on MacConkey agar, catalase positive though some strains may show variation. Various strains of these species participate in metabolism of xenobiotic compounds (Abel *et al.*, 2012).

5.7 CONCLUSION

This research focused specifically on identification of an unknown bacteria isolated from the contaminated waste water and evaluation of its potential use in the degradation of 2,2-DCP. The biochemical and molecular analysis suggested that strain SA1 belongs to *Acinetobacter species*. There is possibility that strain SA1 has potential to grow on 2,2-DCP since various strains from this genus are said to participate in the metabolism of xenobiotic compounds .

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