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Utilisation of 2,2DCP by *Staphyloccocus aureus* ZT and *In Silico* Analysis of Putative Dehalogenase

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Abstract. Halogenated compound such as 2,2-dichloropropionic acid is known for its toxicity and polluted many areas especially with agricultural activities. This study focused on the isolation and characterization of the bacterium that can utilise 2,2-dichloropropionic acid from palm oil plantation in Lenga, Johor and *in silico* analysis of putative dehalogenase obtained from NCBI database of the same genus and species. The bacterium was isolated using an enrichment culture media supplemented with 20 mM 2,2-dicholoropropionic acid as a carbon source. The cells were grown at 30˚C with cells doubling time of 2.00±0.005 hours with the maximum growth at A680nm of 1.047 overnight. The partial biochemical tests and morphological examination concluded that the bacterium belongs to the genus *Staphylococcus* sp.. This is the first reported studies of *Staphylococcus* sp. with the ability to grow on 2,2-dichloropropionic acid. The genomic DNA from NCBI database of the same species was analysed assuming the same genus and has identical genomic sequence. The full genome of *Staphylococcus* sp. was screened for dehalogenase gene and haloacid dehalogenase gene was detected in the mobile genetic element of the species revealed that the dehalogenase sequence has little identities to the previously reported dehalogenases.The main outcome of the studies suggesting an *in situ* bioremediation can be regarded as a natural process to detoxify the contaminated sites provided that the microorganisms contained a specialised gene sequence within its genome that served the nature for many long years. Whether microorganisms will be successful in destroying man-made contaminants entirely rely on what types of organisms play a role in *in situ* bioremediation and which contaminants are most susceptible to bioremediation.

Key words: 2,2-dichloropropionic acid;, palm oil; soil bacteria; *Staphylococcus* sp.; NCBI

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INTRODUCTION

Xenobiotics are foreign to the environment and the diverse application of xenobiotic compound makes it very challenging to degrade by many microorganisms. The biological treatment provides a natural system to treat these toxic chemicals (Daugulis, 2001). A few solutions have been expressed using chemical techniques to treat waste, however the problem remains unsolved either expensive or ineffective in the long term and therefore the preferred way to achieve effective bioremediation is to stimulate microorganisms with nutrients and other chemicals that will enable them to destroy the contaminants at the same time. A common bioremediation system is to rely on microorganisms native to the contaminated sites.

Encouraging them to utilise these toxic chemicals at optimum levels, essential for their metabolism (Atashgahi et al., 2018). The halogenated compound is one example of synthetically made to be used in various applications. For instance these halogenated compound can be used as pesticides to maximize the crop yield and to protect the plants from diseases and pests. Herbicides are known to eliminate targeted weeds that disturbs the growth of the crop that leads to the reduction of crop yield (Au, 2003). One of the herbicides manufactured was Dalapon, 2,2-dichloropropionic acid (2,2-DCP) that has been used to fight against weeds such as quack grass and cattails. The 2,2-dichloropropionic acid (2,2-DCP) consists of three-carbon chain with 2 chloride ions attached to the α-carbon (Figure 1).

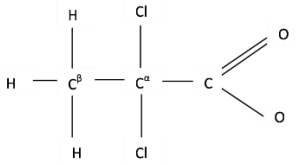

Figure 1. The common structure of 2,2-dichloropropionic acid showing α-carbon and β-carbon that allows halides to attach (Abel et al., 2012).

The presence of 2,2-DCP in the environment can lead to the soil and water pollution and the long term exposure to the chemical can cause an acute side effects (Aparecida et al., 2013). The polluted area by 2,2-DCP transpired when the particle of the 2,2-DCP does not get adsorb within the soil particle that leads to leaching (Holstun and Loomis, 1956). When the herbicide enters the water system, the pollution occurs.

A bacteria has been used to treat 2,2-DCP in soil and this bacteria has been used as the detoxifying agent (Janssen et al., 2005). The bacteria that can degrade halogenated compound possess an enzyme called dehalogenases. The halogenated compound is degraded by a process called dehalogenation. Dehalogenation can happen enzymatically, with the usage of an enzyme to cleave the carbon-halogen bond or by a spontaneous chemical dehalogenation of unstable intermediates (Fetzner & Lingens, 1994). The dehalogenases can be classified into a few categories according to the dehalogenation mechanisms and substrate stereospecificity (Hamid et al., 2011).

The grouping of dehalogenase include Group 1 (stereospecific) and Group 2 (non-stereospecific). Group 1 dehalogenase incudes class 1D and class 2L, whereas Group 2 dehalogenase consist of class 2R only (Harisna et al., 2017). The degradation of 2,2-DCP is carried out through hydrolytic dehalogenation, where the halogen-carbon bond is cleaved by dehalogenase and the chloride ion is replaced by nucleophilic substitution with hydroxyl group derived from water (Ismail et al., 2017). Mechanisms for hydrolytic dehalogenation of 2-haloalkanoic acids was proposed earlier by (Kurihara and Esaki, 2008)

The aims of the present study were to isolate a bacterium that can degrade 2,2-DCP from soil and further characterize its dehalogenase. The analysed dehalogenase gene sequence must be from the same genus and species that was previously isolated. The benefit of the genomic screening of dehalogenases in the database may shed a light that the dehalogenase producing organism are common to a certain microorganisms and important on the aspect of bioremediation.

METHODS

Growth media preparation

The bacterium was grown in a minimal medium supplemented with 2,2-DCP as carbon source. The stock solution was prepared by mixing 10x of basal salts containing K_2HPO_4 (16.22 g), $NaH_2PO_4.2H_2O$ (5.00 g) and $(NH_4)2SO_4$ (12.50 g) with metal salts containing Nitriloacetic Acid (0.5 g), $MgSO_4.7H_2O$(1.00 g), $Fe_2SO_4.7H_2O$ (0.06 g), $MnSO_4.H_2O$ (0.015 g), $ZnSO_4.H_2O$ (0.015 g), $CoCl_2.6H_2O$ (0.005 g) in distilled water (Wong and Huyop, 2011). Minimal media for the bacterial growth contained 10 ml of trace metal and 10 ml of basal salts per 100 ml of distilled water and were autoclaved at 121˚C for 15 minutes at 15 psi. The carbon sources (1 M of 2,2-DCP and 1 M of pyruvate) were sterilized separately and added aseptically to the media to the desired final concentration (Almaki et al., 2016). To prepare solid minimal media, Oxoid bacteriological agar (1.5% w/v) was added prior to sterilization. Samples were removed periodically and optical density reading was taken every two hours for 20 hours period at the absorbance of A680nm.

Sample preparation

Soil sample (5 g) from palm oil plantation from Lenga, Johor was mixed in minimal medium containing 10 mM of 2,2-DCP and incubated in the rotary shaker at 30°C. Bacterial growth was monitored every two hours. For the isolation of pure colonies, 0.1 mL of aliquot was spread onto solid minimal media contained 20 mM 2,2-DCP. For cells purification, colonies were selected and subcultured onto fresh plates containing 10 mM of 2,2-DCP as carbon source. Plates were incubated at 30°C until colonies were formed.

Biochemical tests

A variety of biochemical tests that were carried out including starch hydrolysis tests, oxidase test, catalase test, lactose utilization test, Simmons citrate test, indole test, and urease test to characterize bacteria's biochemical properties. All test media were incubated at a temperature ranging from 30 to 35°C separately to observe their optimum bacterial activity.

Genome analysis

Amino acid sequence of haloacid dehalogenase from *Staphylococcus* sp. was acquired from the National Center of Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). The sequence was derived from the plasmid of *Staphylococcus aureus* strain ST398 with Genbank accession number CEO92199.1. The sequence contained 133 amino acids in total. The sequence of the amino acid is presented in Figure 2.

Amino acid sequence alignment

Six sequences of established dehalogenases were obtained from NCBI and compared to the haloacid dehalogenase from *Staphylococcus aureus* strain ST398 (Figure 2). Multiple sequence alignment and pairwise comparison were carried out to determine the specific location of conserved region of each amino acid.

RESULTS AND DISCUSSION

Isolation of 2,2-DCP degrading bacterium

The growth of mix culture was seen on minimal media agar plate with 10 mM 2,2-DCP as a carbon source. The incubation period was monitored over 10

days at 30˚C (Figure 3A). To accelerate the growth of all colonies, 0.05% yeast extract was added as a supplement in the minimal media. Then, a single colony was isolated by repeatedly streaking onto a fresh plates on the same media preparation. Finally, a single colony was selected for further studies (Figure 3B). A single isolate was named as strain ZT.

```
1   MKFRNIQNED YTVCAKNLVS AYEGAPWYNK WTEKEALLRV EATMSGFNSK GYVIEENNKV 60
61  IAMCIGRIDY YFSGMNQFCI DEFHVIPEFQ KNGVGKKLMN SVSNSLKLDE IYKIFLITGG 120
121 ELAENFKKKK TAS* 133
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Figure 2. The full amino acid sequence of haloacid dehalogenase from the plasmid of *Staphylococcus aureus* strain ST398 (accession number of CEO92199.1)

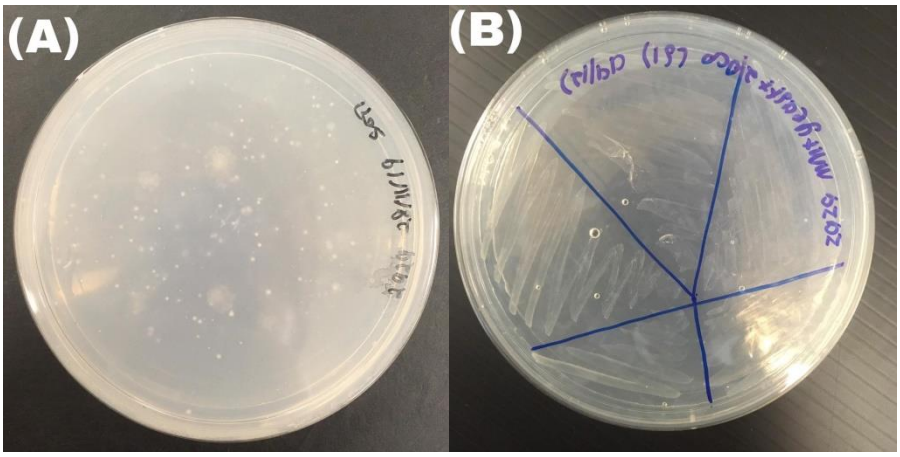

Figure 3. (A) A mix culture from soil were grown on 10 mM 2,2-DCP minimal medium over 10 days period. From this step, a single colony was selected and streaked onto a fresh plate containing the same media component. (B) A selected colony was grown on the same media component with 10 mM of 2,2-DCP and 0.05% yeast after 48 hours of incubation period at 30˚C.

Morphological analysis of strain ZT on solid agar medium

Strain ZT was subjected to basic morphological tests as summarised in Table 1. It was also grown on the minimal media agar with different concentrations of 2,2-DCP to determine the optimum growth condition. Some colonies started to form at 10 mM and 20 mM plates while no single growth seen at 30 mM and 40 mM of 2,2-DCP plates. This suggests at 30 and 40 mM 2,2-DCP was toxic to the cells. The basic morphological analyses of the colonies are summarised in Table 1.

Table 1. The basic morphological characteristics of strain ZT on the minimal media with 10 mM 2,2-DCP incubated for over 24 hours at 30˚C

Parameters	Results
Margin	Entire
Pigmentation	Yellowish
Texture	Mucoid
Shape	Round

Analysis of growth in liquid 2,2-DCP minimal medium

The growth profile was analyzed by inoculating strain ZT into minimal liquid media with different concentrations of 2,2 DCP (10 mM, 20 mM, 30 mM, and 40 mM). The growth profiles were measured at A680nm at every two hours interval for over 20 hours. The growth profiles are shown in Figure 4.

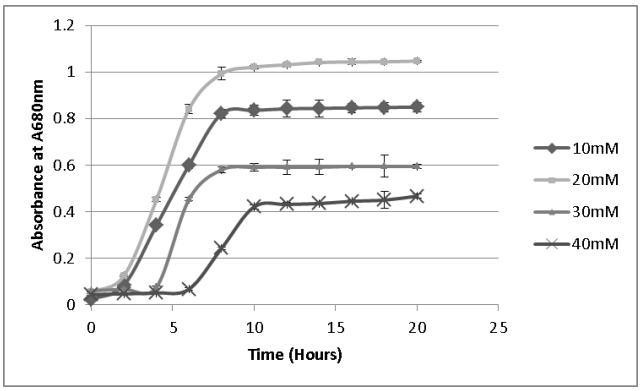

Figure 4. Growth analysis of strain ZT at 10 mM, 20 mM, 30 mM, and 40 mM of 2,2-DCP incubated at 30˚C. Reading were taken in triplicates ± standard deviation.

The growth rates and doubling times of each concentration was observed and calculated. At 20 mM 2,2-DCP showed the best growth condition. The maximum growth was achieved at A680nm of 1.047

with calculated cells doubling time of 2 hours. Meanwhile, growth at 40 mM of 2,2-DCP was the least favourable, as it grows to only 0.467 with unpremeditated doubling time as the cells were presumably dead after 10 hours incubation period. Table 2 summarised the maximum growth of strain ZT in each 2,2-DCP concentration.

Table 2. The maximum growth and calculated cells doubling time of strain ZT in each 2,2-DCP concentration.

Concentration	Maximum growth	Doubling time
10mM	0.849	1.824±0.002
20mM	1.047	2±0.005
30mM	0.595	1.355±0.009
40mM	0.467	NG

Strain ZT showed good growth condition at 10 mM, 20 mM and 30 mM of 2,2-DCP, whereas another study showed that *Acinetobacter calcoaceticus* strain BB9.2 and *Pseudomonas plecoglossicida* strain BC14.3 could grow well in the media containing up to 50 mM and 80 mM of 2,2-DCP (Mulyawati et al., 2018). Most of the previously reported cases showed that 2,2-DCP above 30 mM was toxic to the cells (Oyewusi et al., 2020, Ismail et al., 2017, Edbeib et al., 2017, Muslem et al., 2017, Heidarrezaei et al., 2020). This can be concluded that different bacteria have different mechanisms of uptake of 2,2-DCP and the km values or the affinity of the dehalogenase enzyme. However, *E. coli* could not grow on 2,2-DCP, and this can be attributed to the poor uptake system and/or no expression of the dehalogenase gene in the cells (Jing, *et al.*, 2010).

The dehalogenation product of 2,2-DCP was pyruvate. Therefore, ZT strain was tested to grow in minimal medium containing 10 mM pyruvate as a carbon source. Figure 5 shows ZT strain grew well in pyruvate with cells doubling time of 2.89 hours with a maximum growth of 0.694, whereas the doubling time of strain ZT in 2,2-DCP was 2.85 hours with a maximum growth of 0.624. Likewise, *Rhizobium* sp. RC1 could grow well in 2,2-DCP but not in monochloroacetate (MCA). This attributed to the lacking of an uptake system of MCA into the cells and the product of MCA degradation like glycolate is not a substrate for growth (Huyop and Cooper, 2003).

Identification of ZT strain by biochemical tests

Gram staining was carried out and strain ZT was Gram-positive cocci in clusters (Figure 6). Full results of basic biochemical tests is shown in Table 3. Using the Bergey's manual of systematic bacteriology, it was proposed that strain ZT belongs to the genus *Staphylococcus* sp..

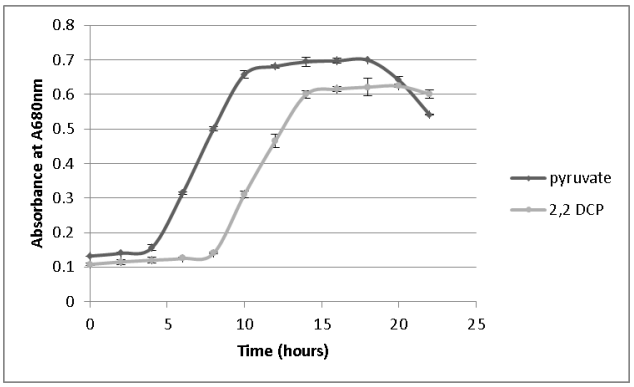

Figure 5. The growth profile of strain ZT in 10 mM liquid minimal media supplemented with pyruvate and 20 mM of 2,2-DCP, at 30˚C. Reading were taken in triplicates ± standard deviation

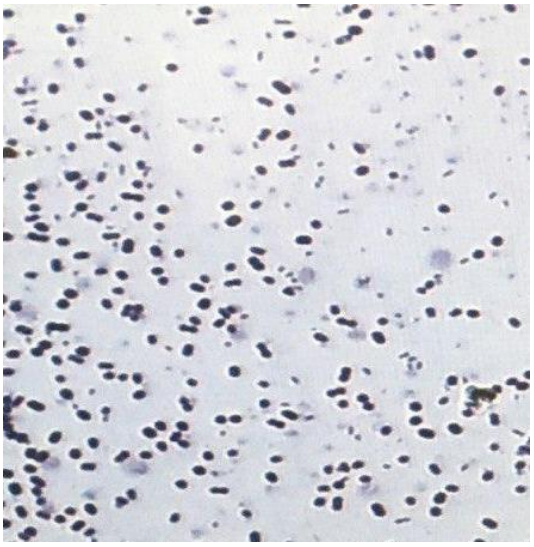

Figure 6. The microscopic observation shows that strain ZT is Gram-positive cocci. (Magnification of 1000x)

Table 3. Basic biochemical tests of strain ZT compared to the biochemical test results obtained from the previous study

Biochemical tests	Results	References (Aggarwal et al., 2012)
Starch test	-	-
Indole test	-	-
Oxidase test	-	-
Catalase test	+	+
Citrate test	+	+
Lactose fermentation test	-	+
Urease test	+	+

(+) indicates positive, (-) indicates negative

Currently, this is the first reported species that can degrade 2,2-DCP. However, according to Camboim et al. (2012), *Staphylococcus* spp. strain ECPB04 can degrade sodium fluoroacetate, which another type of halogenated compound. Another study conducted

revealed that *Staphylococcus xylosus* can degrade 1,2-dichlorobenzene in the presence of glucose (Ziagova and Liakopoulou-Kyriakides, 2007).

Computational analysis

Since *Staphylococcus* sp. ZT strain could grow on 2,2-DCP minimal medium, it was hypothesized that strain ZT possesses dehalogenase enzyme. Therefore, the genome from the gene bank of the same genus of the isolated bacteria was investigated for the presence of putative dehalogenase protein. The amino acid sequence was acquired from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). The sequence was haloacid dehalogenase from the plasmid of *Staphylococcus aureus* strain ST398 (Genbank accession number CEO92199.1; (Fessler et al., 2011). The putative dehalogenase amino acid sequence from strain ST398 was then compared to the common established dehalogenase amino acid sequence obtained from the database to determine the similarities of each haloacid dehalogenases.

The amino acid sequence comparison

The dehalogenase amino acid sequence from different species were selected to be compared with the sequence from the *Staphylococcus aureus* strain ST398 (Table 4). Dehalogenase from strain ST398 was designated as DehST398 (current study).

Table 4. The list of dehalogenases used in comparison with the haloacid dehalogenase from *Staphylococcus aureus* strain ST398 (Genbank accession number: CEO92199.1)

Bacteria	Dehalogenase type	Accession number	Sequence length	References
Klebsiella pneumoniae	Had	ODM40643.1	229	(Zhou et al.)
Rhizobium sp. RC1	DehL	CAA63794	279	(Cairns et al., 1996)
Pseudomonas putida strain AJ1	HadL	AAA25832	227	Barth et al., (1992)
Pseudomonas putida strain 109	DehH109	BAA04474	224	(Kawasaki et al., 1994)
Xanthobacter autotrophicus strain GJ10	DhlB	AAA27590	253	(Van Der Ploeg et al., 1991)
Pseudomonas sp CBS3	DehCI	AAA63640.1	227	(Schneider et al., 1991)

Pairwise sequence alignment

The pairwise comparison was carried out to determine the identities of each dehalogenase against DehST398. The results are presented in Table 5. The sequence identity were less than 10% and somewhat different to each other suggesting DehST398 might serve different functions as the reported amino acid sequences.

Multiple Sequence Alignment

The multiple sequence alignment was carried out for all six amino acid sequences using common MultiAlin version 5.4.1 software (Corpet, 1988). The multiple sequence alignment was carried out to determine the similarities of the location of the conservative region and the arrangement of the sequence. The alignment has a minimum sequence length of 133 and the maximum length of the sequence of 279 with an average sequence length of 224. Figure 7 showed a single conserved region at Arg207 (R207).

Table 5. The pairwise comparison between DehST398 of haloacid dehalogenase *Staphylococcus aureus* strain ST398 with six different haloacid dehalogenases from other species. The identity of each enzyme was less than 10%.

Pairwise		Identity (%)
DehST398	Had	0.9
	DehL	2.6
	HadL	5.3
	DehH109	6.1
	DhlB	2.9
	DehCI	2.4

```
          1                                                  50
 DehST398  .......... .......... .......... .......... ..........
     Deh1  MIRAIVTDIE GTTSDIR.FV HNVLFPYARE RLAGFVTAQQ HAEPVKTILD
     Deh2  ..MSLKKRIK ALTFDTGGTV ARLAVPASEM LLRRQAAGTE SIETGRYWPM
     Deh3  .....MKNIQ GIVFDLYGTL YDV....HSV VQACEEVYPG QGDAISRLWR
     Deh4  .....MQPIE GIVFDLYGTL YDV....HSV VQACESAYPG QGEAISRLWR
     Deh6  .....MDPIR ACVFDAYGTL LDV....NTA VMKHAHDIGG CAEELSSLWR
     Deh5  .......MIK AVVFDAYGTL FDV....QSV ADATERAYPG RGEYITQVWR
Consensus  ........i. ...fd..gt. ..v....... .......... ..e.......

          51                                                 100
 DehST398  .......... .......... .......... .......... ..........
     Deh1  NLRRETDAPA ASTADLITTL FAFMDEDRKS T.ALKALQGI IWRDGYLNGD
     Deh2  NCRRRSMQAM LNLGREPPRH TTLMVRHQFS LDAILAEEGL DVFDDEDRAH
     Deh3  QKQLEYTWLR SLMGRYVNFE KATEDALRFT CTHLGLSLDD ETHQRLSDAY
     Deh4  QKQLEYTWLS SLMGRYASFE QRTEEALRYT CKHLGLATDE TTLRQLGQAY
     Deh6  QRQLEYSWTR TLMGRYADFW QLTTEALDFA LESFGLLERT DLKNRLLDAY
     Deh5  QKQLEYSWLR ALMGRYADFW GVTREALAYT LGTLGLEPDE SFLAGMAQAY
Consensus  ....e..... ...gr..... .......... .......... ........a.

          101                                                150
 DehST398  .......... .......... .......... .......... MKFRNIQNED
     Deh1  FTGHLYPDVL PALEKWKAQG IDLYVYSSGS VAAQKLLFGY SDE.GDITHL
     Deh2  CWDAPHSFDP GDVRDGLARL RDRYIAVSFT FVSHRLIIDT TSVVTGLMWM
     Deh3  LHLTPYADTA DAVRRLKAAG LPLGIISNGS HCSIEQVVTN SEMNWAFDQL
     Deh4  LHLAPHPDTT AALRRLKASG LPMAIASNGS HHSIEQVVSH SDMGWAFDHL
     Deh6  HELSAYPDAV GTLGALKAAG FTTAILSNGN NEMLRGALRA GNLTEALDQC
     Deh5  NRLTPYPDAA QCLAEL..AP LKRAILSNGA PDMLQALVAN AGLTDSFDAV
Consensus  .....ypd.. ..l....a.. ....i.s.g. .......... ..........

          151                                                200
 DehST398  YTVCAKNLVS AYEGAPWYNK WTEKEALLRV EATMSGFNSK GYVIEENNKV
     Deh1  FTGYFDTLVG AKREVQSYRN IAEHLGHAPG TILFLSDIHQ ELDAAEAAGL
     Deh2  RSCLVREWVS TSHCQQICES GGYASRKALR NALWSHAIVS ILMQRETSAS
     Deh3  IS..VEDVQV FKPDSRVYSL AEKRMGFPKE NILFVSSNAW DASAASNFGF
     Deh4  IS..VETVKV FKPDNRVYSL AEQTMAIPRD RLLFVSSNSW DATGARHFGF
     Deh6  IS..VDEIKI YKPDPRVYQF ACDRLDVRPS EVCFVSSNAW DIGGAGAFGF
     Deh5  IS..VDAKRV FKPHPDSYAL VEEVLGVTPA EVLFVSSNGF DVGGAKNFGF
Consensus  .s..v...v. .k.....y.. ..e....... ..lf.s.n.. ....ae..g.

          201                                                250
 DehST398  IAMCIGRIDY YFSGMNQFCI DEFHVIPEFQ KNGVGKKLMN SVSNSLKLDE
     Deh1  RTIQLVRGDR DPASHHPQVQ RFDDIHPEQI PA........ ..........
     Deh2  GQPLINRPDE WGKAIGPQKP P.PGSEPYDI ELNSFLELAA FLESESSLKA
     Deh3  PVCWINRQNG AFDELDAKPT HVVRNLAEMS NWLVNSLD.. ..........
     Deh4  PVCWVNRQGA VFDELGATPT REVRDLGEMS DWLLD..... ..........
     Deh6  NTVRINRINK PQEYSFAPQR HQLSSLSELP QLLLRLTQ.. ..........
     Deh5  SVARVARLSQ .EALARELVS GTIAPLTMFK ALRMREETYA EAPDFVVPAL
Consensus  ....i.R.d. .......... ......pe.. .......... ..........

          251                              282
 DehST398  IYKIFLITGG ELAENFKKKK TAS.......  ..
     Deh1  .......... .......... ..........  ..
     Deh2  NAISGAVRGS AARPTAPGRY ALVALYASAE  GK
     Deh3  .......... .......... ..........  ..
     Deh4  .......... .......... ..........  ..
     Deh6  .......... .......... ..........  ..
     Deh5  GDLPRLVRGM AGAHLAPAV. ..........  ..
Consensus  ........g. .......... ..........  ..
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Figure 7. The multiple sequence alignment of haloacid dehalogenase from *Staphylococcus aureus* (CEO92199.1) (Feßler et al., 2011), *Klebsiella pneumoniae* (ODM40643.1) (Zhou et al.), *Rhizobium* sp. RC1(CAA63794) (Cairns et al., 1996), *Pseudomonas putida* strain AJ1(AAA25832) (Cairns et al., 1996), *Pseudomonas putida* strain 109 (BAA04474) (Kawasaki et al., 1994), *Xanthobacter autotrophicus* strain GJ10 (AAA27590) (Van Der Ploeg et al., 1991), and *Pseudomonas* sp. CBS3 (AAA63640.1) (Schneider et al., 1991)

This is the first reported study on *Staphylococcus* sp. with the ability to grow on 2,2-dichloropropionic acid a model substrate for pollutant degradation. The genomic DNA from the NCBI database of similar bacterial species was analyzed, assuming that the same genus and species have identical genomic sequence. Screening the full genome of *Staphylococcus* sp. for the dehalogenase gene detected the gene in the species' mobile genetic element. The dehalogenase sequence showed low identity to other previously reported dehalogenases.

CONCLUSION

The current study offers new perspectives on the research aside from bioprospecting for new microorganisms. It is also vital that the genomic analysis focuses on the same isolated species when screening potential enzymes and proteins. This study may shed light on the biochemical characteristics of dehalogenases isolated from the bacterial plasmid, with possible bioremediation use. This study isolated the bacterium from an oil palm plantation and identified as *Staphylococcus* sp., which has yet to be reported.

Hence, further investigation into the protein and its 3D structure is necessary to identify the bacteria's conserved region. This study's findings further add to the list of bacteria capable of degrading halogenated compounds, particularly the 2,2-dichloropropionic acid.

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