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Cloning of *dehD* Gene into an Expression Vector pUC19 for Potential Application in Bioremediation

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Abstract
Dehalogenase D (*dehD*) is produced by *Rhizobium* sp. RC1 which breakdown carbon-halogen bond of D-halogenated compounds which are known recalcitrant chemicals. The present research intends to clone *dehD* into a suitable expression vector *pUC*19 for possible use in bioremediation of recalcitrant chemicals such as D-halogenated compounds. *The dehD* gene of *Rhizobium* sp. RC1 sequence was retrieved from GenBank, synthesized by Genscript, USA. The gene construct in pUC57 and pUC19 plasmid were digested using *Bam*HI and *Hind*III restriction enzymes and ligated using Quick T4 DNA ligase which allows ligation of DNA fragments with blunt end or cohesive end within 5 min at 25 ^{O}C. The clone was re-digested using *Bam*HI and *Hind*III and analyzed using agarose gel electrophoresis and sequencing technique. It was established that *deh*D was cloned into *E. coli* using pUC19 vector. This was necessitated in order to obtain dehalogenase enzyme with enhanced potentials in environmental bioremediation, for industrial production of chemicals, pharmaceutical and medical applications. This is the first report on cloning of *dehD* gene for possible application in bioremediation.

Keywords: *Rhizobium* sp. RC1; *dehalogenase; dehD gene; bioremediation.*

Introduction

The use of halogenated compounds in manufacturing and chemical industries for the production of herbicides, insecticides and organic solvents lead to introduction of recalcitrant and toxic metabolites into the environment. Environmental bioremediation of contaminants such as agricultural wastes, sewage and industrial waste products is inevitable. The use of bacterial enzymes has great potential for its low cost and effectiveness. An enzyme D-specific dehalogenase catalyzes hydrolytic dehalogenation of low molecular weight D-specific substrates by attacking their α-carbon atom position using S_N2 hydrolytic substitution reactions to produce inverted configuration of the final product. Mechanistically, the hydrolytic catalysis S_N2 hydrolytic substitutions involves direct attack of the α-carbon atom of the halogenated compound by Asp residue to form ester intermediate and release the corresponding halide ion. The reaction is followed by the attack of Asp residue by activated water molecule at the γ-carbon atom in order to break the ester bond of enzyme product complex (Weightman *et al.*, 2002; Janssen *et al.*, 2001: Hill *et al.*, 1999; Ridder *et al.*, 1999; Slater *et al.*, 1996; Leigh *et al.*, 1986; Leigh *et al.*, 1988; Li *et al.*, 1998; Swanson, 1999).

Organohalogen compounds though they pose a great environmental hazard by interfering with metabolic processes are widely found in nature, with over 700 isolated from prokaryotes and eukaryotes (Gantzer and Wackett, 1991; Fowden and Robinson, 1968). The great physiological and phylogenetic diversity observed may be due to evolutionary pressure on these organisms to colonize every habitat and every microenvironment within each habitat that will support life (Madigan, 2000). The accumulation of the environmental pollutants is mostly attributable to human activities that range from manufacture of chiral intermediates, to recycling of chlorinated by-products from chemical manufacturing, and treatment process of waste streams (Swanson,

1999). Most of these compounds found in the environment are chlorinated organic compounds followed by brominated compounds with few fluorinated and iodinated compounds (Siuda and DeBernardis, 1973).

Some group of microorganisms capable of decomposing chloro-substituted aliphatic acids were isolated from soil by group of dehalogenation enzymes termed "dehalogenases" (Allpress and Gowland, 1998; Bolton *et al.,* 2008; Jensen, 1957; Jensen, 1960) and the same name is used for enzymes involved in dehalogenation of 2-haloakanoic acids (Slater *et al.,* 1995). The study of dehalogenases involved in the conversion of organohalogen compounds have yielded much useful information about catabolic enzymes and their potential applications in environmental technologies and the chemical industry (Allpress and Gowland, 1998; Kurihara, 2011; Metzler, 1977; Mowafy *et al.,* 2010). For the catabolism of recalcitrant halogenated organic compounds in the biosphere, dehalogenation is regarded as the key first step (Hill *et al.,* 1999). The use of dehalogenases in industrial processes is well established (Taylor, 1985), and potentially can be used for the biodegradation of environmentally toxic halogenated compounds such as man-made chemicals used as refrigerants, fire retardants, paints, solvents, herbicides and pesticides (Chaudhry and Chapalamadugu, 1991). Certain dehalogenases initiate the breakdown of halogenated organic compounds by cleaving the carbon–halogen bond with the inversion of the configuration of the chiral carbon (Weightman *et al*., 2002; Huyop and Nemati, 2010; O'Hagan and Schmidberger, 2010; Schmidberger *et al.,* 2008; Schmidberger *et al.,* 2007; Weightman *et al.,* 1982).

Rhizobium sp. belongs to family *Rhizobia*, soil bacteria that normally found in root nodules of leguminous plants. It was initially isolated using 2,2-dichloropropionate (2,2-DCP) as a sole source for carbon and energy by Skinner and his colleagues at Nottingham University (Berry *et al.,* 1979). Leigh *et al*. (1988) and Allison *et al*. (1983) showed that *Rhizobium* sp. was a fast growing, Gram-negative rod microorganism ranging in size from 0.42 to 2.4 µm. This was confirmed using 16S rRNA gene analysis which is in conformity with the earlier report and was designated as *Rhizobium* sp. RC (Huyop and Nemati, 2010). It is against this background that cloning of *dehD* gene is necessitated for potential application in bioremediation of recalcitrant chemicals. The present study is only limited to cloning of dehD gene for further studies in biotechnological applications.

Materials and Methods

Materials

The wild-type *deh*D gene (gi|1103494) of *Rhizobium* sp. RC1 was obtained on NCBI website and synthesized by Genscript, USA. *Escherichia coli* K-12 strain NM522, plasmid pUC19, restriction enzymes *Hind*III and *Bam*HI were obtained from New England Biolabs Inc. Spectrophotometer (Jenway 6300 series) was used. Ampicillin was obtained from Calbiochem, San Diego, California, USA. All other chemicals and reagents used were of analytical grades.

Methods

DehD Gene from Rhizobium sp. RC1, Plasmids and Escherichia coli

The *dehD* gene of *Rhizobium* sp. RC1 sequence was downloaded from GenBank, Sequence alignment using Multalgn (Corpet, 1988) to check gene insertion into bacterial genome and gene synthesized by Genscript, USA. The gene construct in pUC57 contains *Bam*HI and *Hind*III restriction sites. The plasmid pUC19 and *Escherichia coli* K-12 strain NM522 were both obtained from New England Biolabs. All other chemicals used were of analytical grades.

Extraction of genomic DNA

In all DNA extraction procedure, a single bacterial colony forming unit (cfu) was picked using sterile aluminium loop, inoculated into 100 mL LB broth containing ampicillin and incubated at 37 ^{O}C, 200 rpm for overnight (approximately 16 h). From the overnight culture, 2 mL was inoculated at a logarithmic phase with absorbance at A_{280} equals 0.4 to 0.6 (Park *et al*. 2004; Wang and Rossman, 1994) susing UV-visible spectrophotometer (Schimadzu, Japan). The DNA was extracted using QIAprep Spin Miniprep Kit according to the manufacturer's protocol. The DNA concentration was measured using a Nano Drop 1000 Spectrophotometer (Thermo scientific, USA).

***Restriction endonuclease* digestion**

The use of plasmid pUC19 was established using endonuclease restriction analysis and approximate molecular weights determined using agarose gel electrophoresis. Restriction analysis was carried out using high fidelity *Bam*HI (*Bam*HI- HF™) and *HindIII (HindIII*- HF™) restriction endonucleases (NEB). The reaction contained 1 x NEB buffer, 3 µL sample DNA and pUC19, 1 µL each of restriction enzyme when performing double digest and 9 µL nuclease-free water to make up a 15 µL final volume. The mixture was flicked 3 to 5 times, spin at 13,000 x g for 5 sec and incubated at room temperature for 1 hour. *Hind*III-HF™ requires heat inactivation at 80 ^{O}C for 20 min while *BamH*I-HF™ does not require heat inactivation. Successful restriction digestion was analyzed using agarose gel electrophoresis (Sambrook and Russell (2006).

Ligation strategy

Restriction endonuclease digested plasmid DNA were re-circularized using Quick T4 DNA ligase (NEB) as described by the manufacturers. Quick T4 DNA ligase allows ligation of DNA fragments with blunt end or cohesive end in 5 min at 25 ^{O}C. T4 DNA ligase does not require heat inactivation since such reduces transformation efficiency. 2,5 µL digested DNA vector was added to aliquots of 2x Quick ligation buffer, 7.5 µL double deionized water, 1 µL Quick T4 DNA ligase and mixed thoroughly by flicking before incubation for 5 min at room temperature and chill on ice. The mixture was transformed to confirm ligation or stored at -20 ^{O}C for further use. The success in transformation was further determined using agarose gel electrophoresis and subsequent sequencing.

Transformation of E. coli

Competent *E. coli* cells were prepared from overnight culture of *E. coli* stock grown at 37 ^{O}C with shaking at 200 rpm was inoculated into 100 mL LB. Bacteria grown at OD_{600}, 0.30-0.95 were placed on ice for 15 min and centrifuged at 4000 rpm (in Sorvall 5C R Plus centrifuge) at 4^{O}C for 10 min. The pellet was suspended in 30-40 mL 0.1 M $CaCl_2$ solution and allowed to chill on ice for 30 min. Centrifugation was repeated as earlier described and pellet re-suspended in 6 mL of 0.1 M $CaCl_2$ containing 15% glycerol. Into sterile 1.5 mL mirocentrifuge tubes were added aliquots of 0.5 mL cell suspensions and freeze dried on ice (Nakata, Tang et al. 1997; Hansen, Knudsen et al. 1998; Sambrook and Russell 2006). Overnight culture of *E.coli* competent cells was inoculated into LB broth containing ampicillin and used for transformation. Competent *E.coli* cells were thawed on ice. 3 µL of plasmid (pUC18/19) was allowed to chill in 1.5 mL centrifuge tube and 3µL plasmid DNA to competent cells added, the tubes flicked 4-5 times in order to mix the cells and plasmid DNA (without votexing). The mixture was placed on ice for 30 min (without mixing) and heat shocked at 42^{O}C for 20 sec (without shaking). 250 µL of SOC (37^{O}C) media was added into the tube for 60 min. The mixture shaked vigorously (300 rpm) using Thermomixer and serially diluted 10-fold in SOC before spreading 50-100 µL of each dilution onto a selection plate and incubated overnight at 37^{O}C. Transformation of *E. coli* cells were determined using blue-white screening 5-bromo-4-chloro-indolyl-D-galactoside (X-gal) according to standard methods. Bacterial cell colonies that appear blue due to genetic alteration are considered transformed cells (Militello *et al.,* 2016; Calos, *et al.,* 1983) and selected for further use.

Agarose gel electrophoresis

Agarose gel was composed by adding 0.4 agarose into 1 x TAE buffer (50 mL) and mixture heated for 1 min, clear solution was made to cool before adding 0.5 µg/mL ethidium bromide into clear gel solution. Then it was poured into the casting tray containing appropriate comb to create wells and left to solidify. After solidification, the comb was removed from the gel. And the solidified gel was removed from the casting tray and placed in an electrophoresis tank containing 1x TAE buffer pH 7.6. Then 1µg of 1 kb DNA ladder (Promega) and 2 to 5 µL samples containing 1x loading dye were dispensed into the wells in the gel. The electrophoresis separation of the sample constituents was carried out at 80 volts for 1 h.

The UV transilluminator was used to visualize and capture the separated bands on the gel. The sizes of the bands were estimated by comparison with 1 kb ladder. Fragments of interest were excised with sterile scalpel blade under UV illumination and placed into 1.5 mL Eppendorf tube and the DNA extracted using Qiagen gel extraction kit (Qiagen Hilden, Germany).

Results and Discussion

Figure 1 shows the result of vector and insert analysis of the dehalogenase D *(dehD)* from *Rhizobium sp.* RC1. The *dehD* gene construct in pUC57 was made from plasmid pUC57 (2710 bp) originally contain *dehD* gene (798 bp) incorporated with *Bam*HI and *Hin*dIII restriction sites which was designated pUC57 *dehD*$^{+}$.

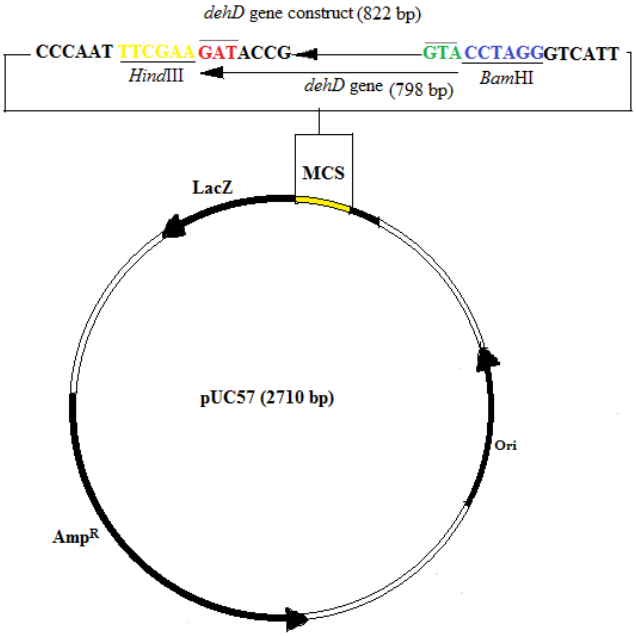

Figure 1: The pUC57 *dehD*$^{+}$ construct restriction sites. Start codon in *dehD* is shown in green and stop codon in red color.

Figure 2 shows the result of analysis *pUC19 dehD*$^{+}$. Double digestion of pUC57 *dehD*$^{+}$ showed two distinct bands on agarose gel of approximate sizes of 2,710 bp and 798 bp equivalents to the lengths of pUC57 DNA and *dehD* gene, respectively. Figure 3 further confirmed the presence of *dehD* in the recombinant plasmid, the miniprep product was sequenced and compared to the sequence of *dehD* in the data base (Cairns *et al.*, 1996). The result also showed identical sequence to *dehD* gene sequence in the data base. Therefore, pUC57 *dehD*$^{+}$ plasmid certainly contained *dehD* gene and was further used in this research.

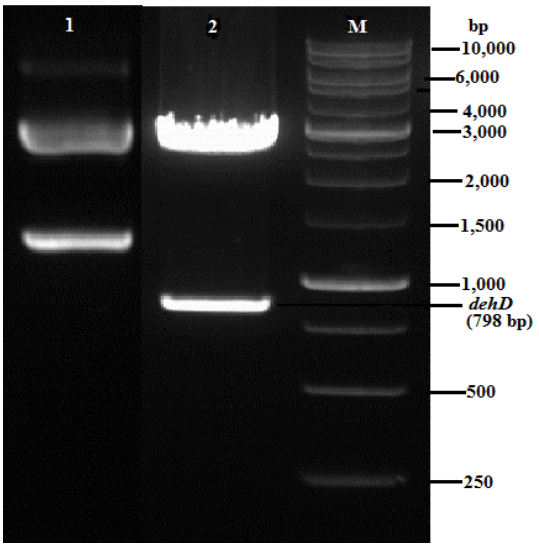

Figure 2: Restriction enzyme digestion of pUC19 *dehD*$^{+}$ plasmid. Lane 1 shows undigested sample, lane 2 is fragments from *Bam*HI and *Hin*dIII digest, and M represent DNA ladder.

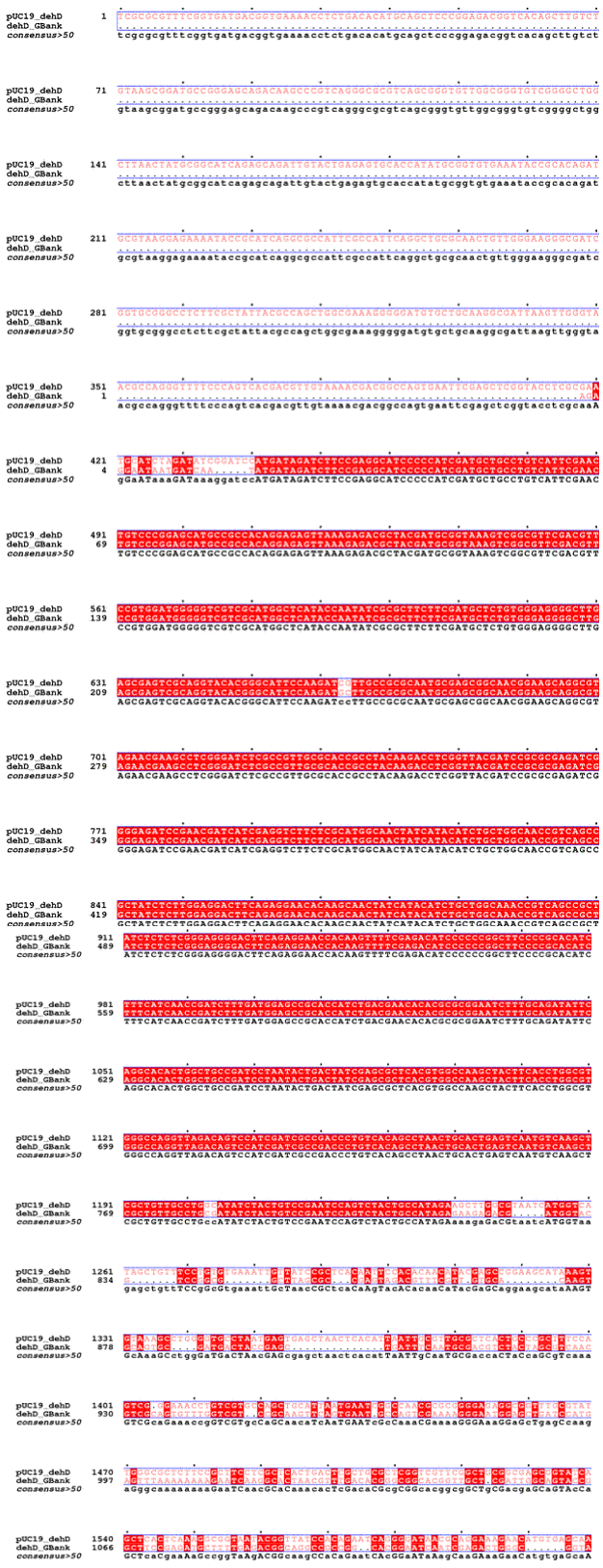

Figure 3: Pairwise sequence alignment of pUC19 *dehD*$^{+}$ and *deh*D from GenBank id: X93597.1 contained in *Rhizobium* sp. *deh*D and *dehL* genes (X93597) (Cairns *et al.*, 1996). Only 1-1610 nucleotides are shown in pUC19 *dehD*$^{+}$ and only 1-1136 nucleotides shown in *deh*D. Sequences of *deh*D starts from nucleotide number 441 and stops at position 1239 shown in red background with white characters.

Figure 4 shows the construction of *dehD gene into pUC19.* Plasmid gene, pUC19 was cut with *Bam*HI and *Hin*dIII endonuclease restriction digest showing 2.7 kb band.

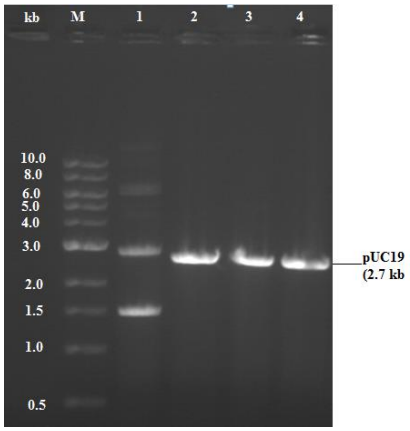

Figure 4: Confirmation of pUC19 plasmid using restriction enzymes**.** Lane M is Ladder, lane 1 lane is *Bam*HI and *Hin*dIII double digest. Lane 2 is undigested pUC19, lane 3 is *Hin*dIII digest, and lane 4 is *Bam*HI digest.

Figure 5 shows the confirmation of cloned *dehD in pUC19.* The *dehD*$^+$ gene cut with *Bam*HI and *Hin*dIII from pUC57 was ligated into pUC19 also cut with the same restriction enzymes; the product was designated pUC19 *dehD*$^+$. The pUC19 *dehD*$^+$ was analysed using *Bam*HI and *Hin*dIII restriction endonucleases digestion which was separated using agarose gel electrophoresis.

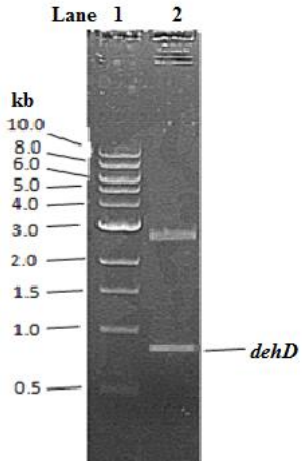

Figure 5: Confirmation of ligation of *dehD* into pUC19 using *Bam*HI and *Hin*dIII restriction digest analysis. Lane 1 DNA ladder, and Lane 2 *Bam*HI and *Hin*dIII double digest of recombinant pUC19 carrying *dehD* gene.

Figure 6 shows the sequence analysis of pUC19 *dehD*$^+$. The restriction enzyme analysis of pUC19 *dehD*$^+$. Sequence analysis of pUC *dehD*$^+$ using Multalign (Corpet, 1988) was carried out in order to check the ligation and in section of *dehD*$^+$ in the expression vector. The result for sequence of pUC19 *dehD*$^+$ is presented (Figure 6) showing only the first 2941 bp. The result shows that *dehD* (in yellow back ground color, restriction sites; *Hin*dIII isshown in red background with white characters and *Bam*HI is shown in *blue* background with white characters) have been included in the recombinant protein. Figure 6 showed a ligation protocol of the insert in the vector with appropriate start and stop codon.

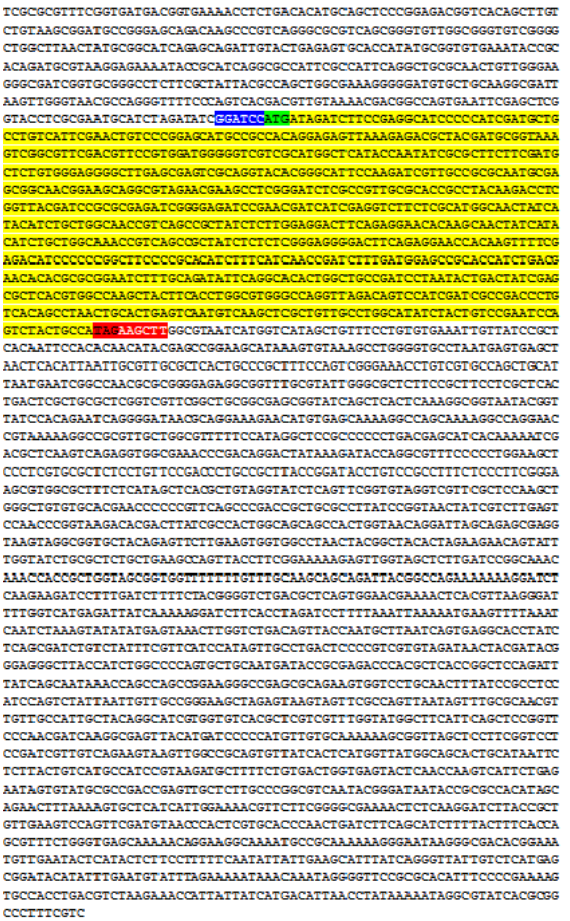

Figure 6: Sequence analysis of pUC19 *dehD*$^{+}$ DNA plasmid. Green background with black character is start codon; red background with black character is stop codon; red background with white character is *Hin*dIII restriction sites; blue back ground with white character is *Bam*HI restriction site.

The double digestion of pUC57 *dehD*$^{+}$ with *Bam*HI and *Hin*dIII restriction endonucleases gave two distinct bands on agarose gel with approximate sizes of 2,710 bp and 798 bp equivalents to molecular weights of pUC57 DNA and *dehD* gene, respectively; confirming the presence of *dehD*. To further con firm the presence of *dehD* in the recombinant plasmid, the miniprep product was sequenced and compared to the sequence of *dehD* in the data base (Cairns *et al.,* 1996) (Figure 3). The pairwise sequence analysis showed identical sequence to *dehD* gene sequence in the data base. Therefore, pUC57 *dehD*$^{+}$ plasmid certainly contained *dehD* gene and was used in this sub-cloning research.

The *Bam*HI and *Hin*dIII restriction digest (Figure 5), indicates that the pUC19 *dehD*$^{+}$ contained two fragments. The two fragments are approximately 2,686 bp and 798 bp in size. The sequence when compared with that *dehD,* showed identity to each other. The 2,686 bp fragment represents pUC19 vector gene and 798 bp corresponds to *dehD* gene. This indicate that the clone was successful, hence could be used for further research bearing in mind the computationally resolved key catalytic residues of *dehD* (Sudi *et al.*, 2014; Sudi *et al.,* 2014).

Conclusion

In conclusion, the *dehD* gene was successfully cloned into the pUC19 expression vector to give pUC19 *dehD*$^{+}$ gene. The final construct designated pUC19 *dehD*$^{+}$ harbors the *dehD* gene 798 bp in length. The gene construct also includes *Bam*HI and *Hin*dIII restriction sites. The clone could be exploited for potential bioremediation, industrial production of chemicals, pharmaceutical and medical applications.

X-ray crystallization of *deh*D protein is worth trying to further ascertain the roles played by their respective active site amino acid residues. There is

also a need for future work on site-directed mutagenesis of the key catalytic residues of *deh*D in order to ascertain their involvement in dehalogenation processes.

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Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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