Research article

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Isolation and Characterisation of the Degradation Potential of 2,2-Dichloropropionate (2,2-Dcp) By *Bacillus amyloliquefaciens* From Gebeng

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ABSTRACT

Most halogenated compounds such as α-halocarboxylic acid (αHCA) are potential carcinogens that are toxic and widely liberated in both aquatic and terrestrial ecosystem. 2,2-dichloropropionate (2,2-DCP) is a synthetic halogenated compound commonly used in agricultural activities as an herbicide. This study is focused on the isolation and characterization of dehalogenase producing bacteria capable of utilizing 2,2-dichloropropionate as the sole carbon and energy source from soil sample obtained from the industrial hub of Gebeng (Pahang), Malaysia. An enrichment culture technique supplemented with 2,2-DCP was used to isolate the bacteria. A pure culture strain designated as strain FDS grew well in media containing 20 mM 2,2-DCP at a temperature of 30˚C exhibiting a cell doubling time of 22.21hours and a maximum rate of chloride ion release of 0.345mmol/L suggesting this is the optimum concentration for growth. Based on microscopic observations, biochemical characteristics and phylogenetic analysis of the 16S rRNA gene sequence, FDS was identified as *Bacillus amyloliquefaciens* with 98% homology. A literature survey revealed no report regarding the role of *B. amyloliquefaciens* on pesticide biodegradation as well as on 2,2-DCP, therefore the current isolate was designated *Bacillus amyloliquefaciens* FDS.

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Introduction

The advancement in technology has resulted in the formation of various man-made organic compounds. These compounds (xenobiotics) are produced in enormous quantities which also benefit our life. Among these xenobiotics are halogenated compounds. These

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compounds are extensively used as antibiotics (chloramphenicol, vancomycin), anticancer (iododoxorubicin, salinosporamide) and as agrochemicals (herbicides, pesticides, soil fumigants) and solvents (lubricants, silicones) [1]. Halogenated compounds have been reported to cause serious health issues such as liver necrosis, dissociation of hepatocytes, fat disintegration [2], hormonal imbalance, lower respiratory problems, skin and lung cancer etc. [3], in addition to causing serious environmental pollution owing to their toxicity, their toxic breakdown products, persistence and bioaccumulation in the food chain and water bodies [4]. These compounds are poisonous and very difficult to degrade [5].

2,2 -Dichloropropionate (2,2-DCP) is a chlorinated aliphatic acid also known as Dalapon. It is used in agriculture as an herbicide and a plant growth regulator used on certain annual and perennial grasses. It kills only certain plants while sparring non-target plants thereby preventing lipid synthesis, stops cell division, reduces wax production by leaves, affects carbohydrate, lipid and nitrogen metabolism [6].

Microorganisms play an important role in the degradation of xenobiotics, they help transform toxic compounds into non-hazardous substances. Some groups of bacteria use halogenated organic compounds as their main carbon and energy source, these microorganisms detoxify or remove pollutants owing to their diverse metabolic capabilities thereby helping reverse the effect of environmental halogen- associated pollution [7].

These bacteria produce enzymes known as dehalogenase which catalyze the cleavage of carbon-halogen bonds in halogenated organic compounds thereby producing some environmental friendly products by a process known as dehalogenation [8], [9]. Some examples of aerobic degradative bacteria include; *Pseudomonas, Bacillus, Micrococcus* and *Rhodococcus* while anaerobic degradative bacteria are *Syntrophus*, *Methanospirillum*, *Syntrophobacter*. Among them *Pseudomonas* and *Bacillus* species are the most studied [10], [11]. The present study is focused on the isolation and characterization of 2,2-DCP degrading bacteria from Gebeng industrial area using enrichment techniques.

Materials and Methods

Isolation and identification of 2,2-DCP degrading bacteria

The soil sample was collected from the industrial area of Gebeng (Pahang), Malaysia. The industries around the are generate a lot of chemical waste containing halogen, which contaminate soil in the environment.

Preparation of medium for isolation

Varying concentrations (10 mM, 20 mM, 30 mM and 40 mM) of 2,2-DCP liquid minimal medium were prepared according to Table 1. tenfold of trace metal salts which comprised of Nitriloacetic acid (1.0g/L), $MgSO_4.7H_2O$ (2.0g/L), $FeSO_4.7H_2O$ (0.12g/L), $MnSO_4.4H_2O$ (0.03g/L), $CoCl_2.6H_2O$ (0.01g/L), $ZnSO_4.H_2O$ (0.03g/L) were dissolved in distilled water. The basal salts were prepared as a tenfold concentration of $K_2HPO_4.3H_2O$ (42.5g/L), $NaH_2PO4.2H_2O$ (10.0g/L), $(NH_4)_2SO_4$ (20.0g/L) in distilled water. The medium for isolation contained 10ml of 10x trace metal salts and 10ml of 10x basal salts per 100ml distilled water were autoclaved (121˚C, for 15min at 15psi). Then 2ml of 2,2-DCP (1M) was filter sterilized using a nylon filter (0.2µm pore size) and added aseptically into the media and made up to the desired final concentration the plates were prepared by adding bacteriological agar (1.5% w/v) prior to sterilization[12].

Table1 Composition of minimal media

2,2-DCP concentration	**10mM**	**20mM**	**30mM**	**40mM**
Distilled water	79	78	77	76
Trace metal	10	10	10	10
Basal Salt	10	10	10	10
V1, 1M	1	2	3	4
Total (ml)	100	100	100	100

Isolation procedure

5g of sample was added into a 250ml shaker flask containing 100ml minimal salts and supplemented with 20 Mm 2,2-DCP as the sole carbon and energy source. Following 10

days incubation with shaking at 200rpm at 37˚C, an aliquot (0.1ml) was pipette out and spread on plates supplemented with 20 Mm 2,2-DCP and incubated at 37˚C. The resulting colonies were selected and screened. Pure cultures, the resulting colonies were repeatedly sub cultured in the same medium to confirm carbon source utilizing ability.

Identification of isolate

To ascertain the identity of the isolate, Gram staining, motility, spore staining and biochemical test were carried out: Oxidase, catalase, gelatin liquefaction, lactose fermentation, citrate test, Nitrate reduction, indole, urease and starch hydrolysis test [12].

Growth profile

The growth profile of the bacterial culture in different concentrations of 2,2-DCP (10mM-40mM) were monitored by taking 1ml of the sample from the growth medium at 2 hours interval over 20 hours growth period and measured at A_{680nm} using Jenway 6305 UV Spectrophotometer (Staffordshire, UK).

Halide ion assay

The utilization of 2,2-DCP by the isolates results in the release of halide ion during dehalogenation reaction as carried out from adaptation of [13]. A sample (1ml) from the liquid growth medium, 100µl of Reagent I (0.25M ferric ammonium sulfate in 9M nitric acid) was added and thoroughly mixed. To this, Reagent II (100µl of mercuric thiocyanate dissolved in saturated ethanol) was also added and the solution was mixed by shaking. Color could develop for 10 minutes and measured at A_{460nm}.

16S rRNA genomic analysis

The genomic DNA was extracted from an overnight bacterial culture using Promega® Wizard® Genomic DNA Purification Kit (Promega,Madison, WI, USA). The purification and merging the DNA concentration was measured by Nanodrop machine.The Polymerase chain reaction (PCR) was carried out using universal primers, Fd1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP1 (5'-ACG GTC ATA CCT TGT TAC GAC TT-3') to amplify the DNA fragment as suggested by [14], [15]. The PCR cycle for DNA amplification was performed for 30 cycles and set as initial denaturation at 95˚C for 5 minutes; cooling, denaturation at 94˚C for 1 minute; annealing at 55˚C for 1 minute; final

elongation at 72˚C for 10 minutes. The PCR products were purified using QIA quick PCR purification kit and sequenced by 1^{ST} Base Laboratory, Malaysia.

Phylogenetic study

The bacterial 16S rRNA gene sequences were compared with other sequences of dehalogenase producing bacteria in GenBank database using BLASTn. The FASTA format of all 16S rRNA sequences obtained were aligned together by MUSCLE using MEGA7 [16]. The topology of the tree was constructed using neighbor-joining method with bootstrap test values above 50% based on 500 resampling.

Results

Isolation of 2,2-DCP degrading bacteria

Following 5 days incubation of the soil sample obtained from Gebeng industrial area lead to the isolation of a bacterium designated strain FDS. The Size, morphology (Table 2) while Gram staining showed the microorganism to be Gram positive rod shape (figure 1). However, to fully ascertain the identity of strain FDS, biochemical tests (Table 3) were performed according to Bergy's Manual of Systematic Bacteriology [17].

Table 2 Morphological characteristics of Strain FDS on solid media

Parameters	Results
Pigmentation (pure colony)	Creamy
Elevation	Flat
Colony	Rough circular
Margin of the colony	Undulate
Microscopic shape	Rod
Size	3.40µm
Spore formation	Negative
Oxygen requirement	Aerobic

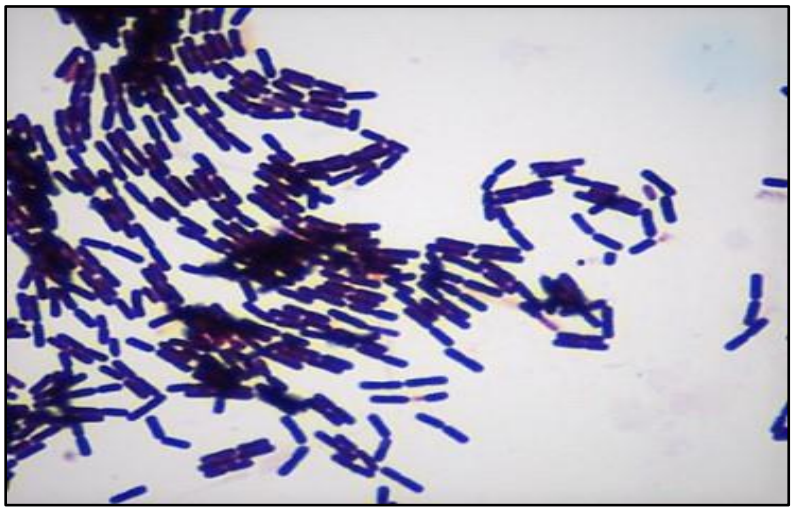

Fig 1 Microscopic observation showing the Gram-stain of strain FDS (1000 X magnification

Table 3 Results from the Partial biochemical tests

Test	Results
Oxidase	Positive
Catalase	Positive
Indole	Positive
Gelatin liquefaction	Positive
Motility	Negative
Citrate	Negative
Urease	Positive
Nitrate reduction	Positive
Starch hydrolysis	Positive
Lactose utilization	Negative

Growth profile and Halide ion assay

The growth profile of FDS in different concentrations (10mM-40mM) of 2,2-DCP are shown (Figure2). 20mM 2,2-DCP exhibited good bacterial growth. 10mM and 30mM 2,2-DCP showed low growth, while 40mM 2,2-DCP exhibited no growth which suggests that the concentration was toxic. Following this observation, the bacterium FDS was grown in

liquid minimal medium supplemented with 20mM 2,2-DCP at 37˚C with agitation at 200 rpm with growth measured at 2 hours interval over 20 hours period. The doubling time of the bacterium was 22.21 hours with a maximum chloride ion of 0.345mmol/L.

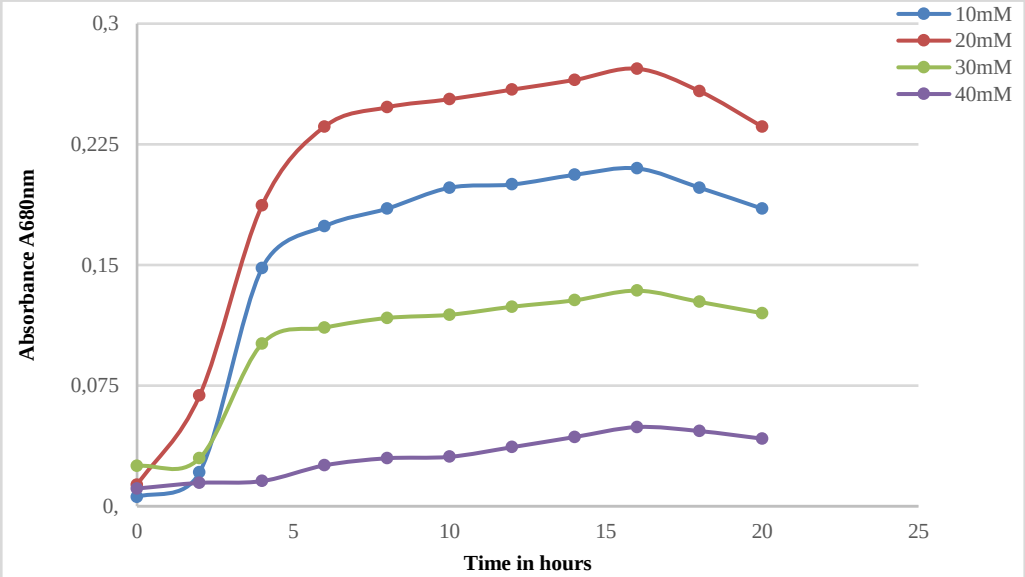

Fig 2 Growth rate of strain FDS in different concentrations of 2, 2-DCP

Bacterial growth on other halogenated compounds

The bacterium FDS was grow in 10mM trichloroacetic acid (TCA), 10mM 2-chloropropionate (2-CP) and 10mM 2-bromopropionate (2-BP) liquid minimal medium. The results (Table 4) showed growth in 10mM TCA while no growth was observed in 2-CP and 2-BP which suggests the substrate is not suitable for growth. The cell doubling time of FDS grown in 10mM TCA was 14.09 hours and a maximum chloride ion of 0.223mmol/L (Figure 3).

Table 4 Growth properties of strain FDS in different substrate

Growth Substrate (10mM)	Maximum Medium Turbidity ($A680_{nm}$)	Doubling time(hours)
Trichloroacetic acid	0.184	14.09
2-bromopropionate	NG*	NG*
2-chloropropionate	NG*	NG*

*NG: No Growth

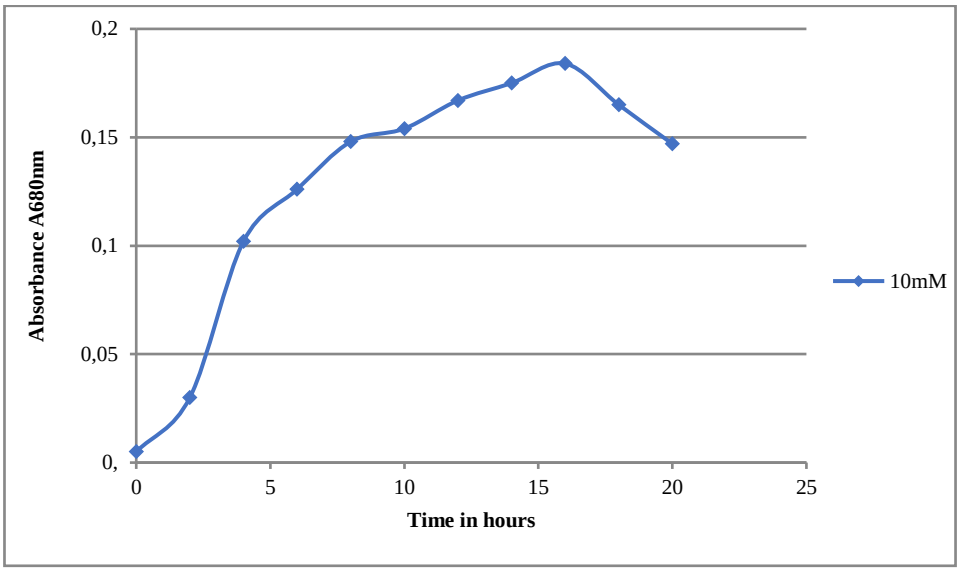

Fig 3 Growth rate of strain FDS in 10mM concentration of TCA

Identification of 2,2-DCP degrading bacteria using 16S rRNA analysis

The genomic DNA of FDS was successfully extracted and amplified using universal primers Fd1 and rP1. Gel electrophoresis of the PCR product revealed a single fragment of approximately 1500bp (Figure 4).

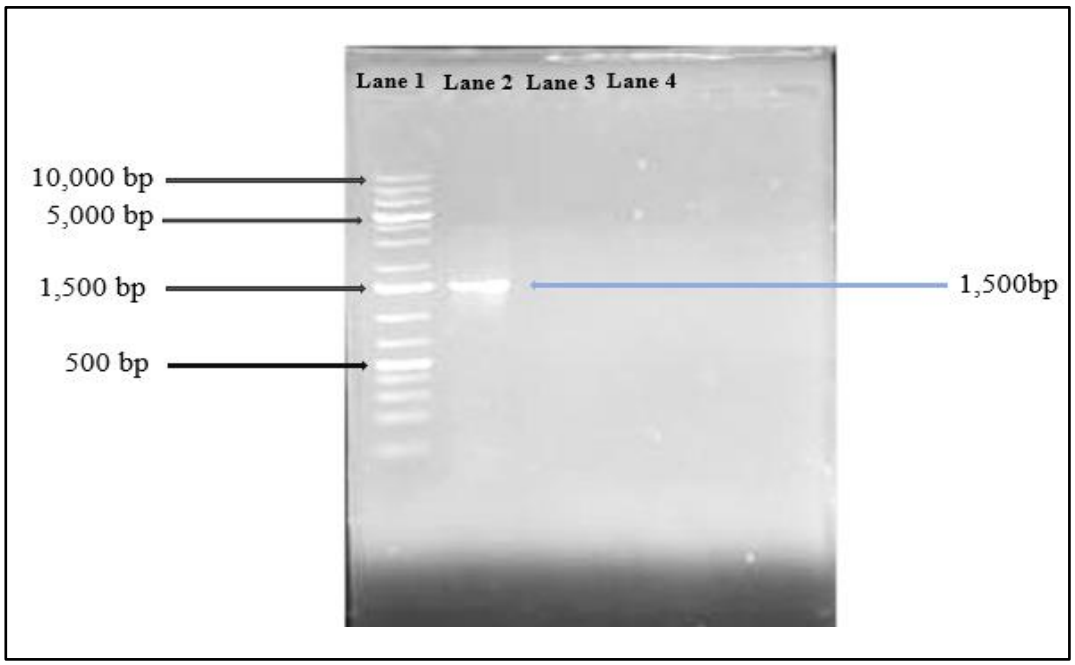

Fig 4 PCR product for 16S rRNA under UV light

Lane 1: 1kb DNA ladder, Lane 2: PCR product of 16S rRNA, Lane 3: Control (dH_2O + Fd1+ PCR mix), Lane 4: Control (dH_2O + rP1+ PCR mix).

Sequencing and analysis of 16S rRNA gene

The outcome of the PCR product sequenced by 1st Base Company was received in ". abl'' format. The files were viewed using Sequence scanner software. The BLASTn results showed that bacterium FDS shared 98% identity to *Bacillus amyloliquefaciens* (Table 5).

Table 5 The top ten BLAST search result of strain FDS

Accession number	Description	Maximum score	Query coverage (%)	Identity (%)
NC 014551.1	*Bacillus amyloliqueficiens* DSM7	1995	97	98
NC 000964.3	*Bacillus subtilis* strain 168	1973	97	98
NZ CP007640.1	*Bacillus atrophaeus* subsp. globiqii strain BSS	1962	97	97

Phylogenetic study

The top dehalogenase producing bacteria obtained from BLASTn analysis as obtained from NCBI database were selected for the construction of phylogenetic tree using MEGA 7 by neighbour-joining with bootstrap value (Figure 5). The results inferred that the bacterium FDS may be designated as *Bacillus amyloliquefaciens* FDS.

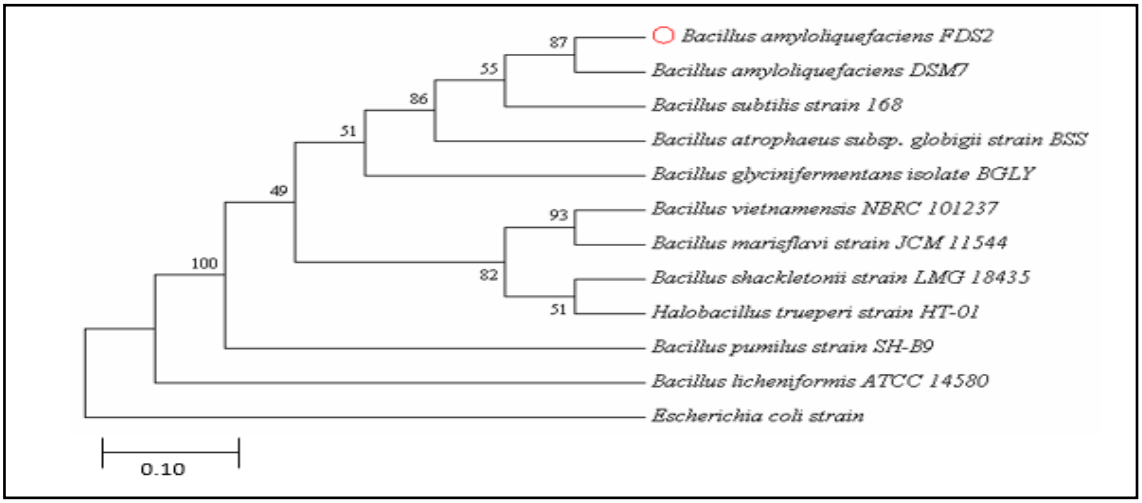

Fig 5 Phylogenetic relationships between *Bacillus amyloliquefaciens* FDS and other dehalogenase producing bacteria16S rRNA sequences

Discussion

In this study, Soil sample obtained from Gebeng industrial area which is synonymous with the manufacturing of various chemical and petrochemical products was used as the source for the isolation of bacteria capable of degrading 2,2-DCP and TCA as the sole carbon and energy. The isolation procedure employed in this study entailed the enrichment of minimal salts with the carbon sources (2,2-DCP and TCA), followed by plating on a similar agar medium. The two procedures help ensures that the isolation is that of bacteria only capable of utilizing the carbon sources. The successful isolation of a strain designated FDS was observed to have various morphological (Table 4.1) and biochemical characteristics (Table 4.2). However, to properly characterize and identify the strain a method of bacterial taxonomy known as 16S rRNA was employed this routine procedure in prokaryotic taxonomy results in large and growing databases which improve identification results, phylogeny reconstructions and primer specificity evaluations[18]. The 16S rRNA gene sequence analysis suggests that strain FDS gene sequence shared at least 98% identity to the sequence of *Bacillus amyloliquefaciens* which suggests strain FDS belongs to the *Bacillus* sp. (Figure 4.9). Thus the 2, 2-dichloropropionate degrading bacteria isolated belong to *Bacillus* sp. This was further supported by staining properties and biochemical analysis and in accordance with Bergy's Manual of Systematic Bacteriology [17]. *Bacillus amyloliquefaciens* is a gram positive, rod-shape, catalase positive and aerobic soil bacteria. As with other members of the *Bacillaceae* they have the ability to form endospore allowing them to survive for a prolonged period time, thereby increasing their soil shelf life [19], [20]. Previous studies have shown that several strains of the bacteria belonging to the genus *Bacillus* particularly *B.subtilis* and *B.amyloliquefaciens* species were known to be effective for the biocontrol of many plant-diseases caused by soil borne pathogens [21], [22] or post-harvest pathogens [23]–[25]. Their ability to colonize plants rhizosphere thereby suppressing competing phytopathogenic bacteria and fungi these classifies them as microbial biopesticides. They represent about half of the commercially available bacterial biocontrol agents [26]. However, there has not been any report of *Bacillus amyloliquefaciens* that is able to degrade 2,2-dichloropropionate and trichloroacetate

reported so far. Therefore, this strain of *Bacillus amyloliquefaciens* that can degrade 2,2-dichloropropionate deserves more research.

They are four basic criteria for a given halogenated compound to be utilized by an organism as a sole carbon and energy source as reported by [27]. Firstly, the organism should be able to synthesize enzyme (dehalogenase) which can remove the substituent halogen(s) from the compound. Secondly, the product of dehalogenation should be non-toxic and easily converted to an intermediate which can readily be absorbed in the organism's central metabolic pathway. Thirdly, entry of halogenated compound into the organism should be either through passive or active transport in order for the compound to be synthesized by the enzyme, and finally, the halogenated compound should not be toxic to the organism at intracellular concentrations [27]. These criteria were satisfied when *Bacillus amyloliquefaciens* FDS grew on 2,2-dichloropropionate and trichloroacetic acid as the sole carbon and energy source. The isolate grew more rapidly on 2,2-dichloropropionate compared to trichloroacetic acid, possibly because the organism was originally isolated using 2,2-dichloropropionate. 2,2-dichloropropionate failed to support growth at concentration exceeding 40mM, while trichloroacetic acid failed to support growth exceeding 10Mm; presumably the intracellular concentration of halo-aliphatic acid had reached a toxic level.

Conclusion

The successful isolation identification and characterization of *Bacillus amyloliquefaciens* FDS capable of degrading 2,2-DCP and TCA from Gebeng would suggest that these study areas possess enormous reservoir of unexplored/unexploited microbes. In conclusion, this study provides the identity of a bacterium that can utilize halogenated compound and used in bioremediation strategies. In future, the study can help describe the diversity of microbial dehalogenases and the dehalogenation enzyme regulation.

Abbreviations

2,2-DCP: 2,2-dichloropropionic acid, BLAST: Basic Local Alignment Search Tool, Br: Bromine, Cl: Chloride, DNA: Deoxyribonucleic acid, EDTA: Ethylenediaminetetraacetic acid, EtBr: Ethidium Bromide, F: Flourine, I: Iodine, LB: Luria Bertani,MEGA: Molecular Evolutionary Genetics Analysis, NaCl: Sodium chloride,NADH: Nicotinamide adenine dinucleotide,NCBI: National Centre for Biotechnology Information, PCR: Polymerase chain reaction, RNA: Ribonucleic acid, sp.: Species, TAE: Tris acetate EDTA, TCA: Trichloroacetic acid, UV: Ultraviolet

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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