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Isolation and Characterization of a Novel Bacterium from the Marine Environment for Trichloroacetic Acid Bioremediation

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Abstract: Halogenated compounds are an important class of environmental pollutants that are widely used in industrial chemicals such as solvents, herbicides, and pesticides. Many studies have been carried out to explore the biodegradation of these chemicals. Trichloroacetic acid (TCA) is one of the main halogenated compounds that are carcinogenic to humans and animals. The bacterium was isolated from the northern coastline of Johor Strait. In this study, the ability of strain MH2 to biodegrade TCA was evaluated by a growth experiment and dehalogenase enzyme assay. The growth profile of the isolated strain was examined. The doubling time for *L. boronitolerans* MH2 was found to be 32 h. The release of chloride ion in the degradation process was measured at $0.33 \times 10^{-3} \pm 0.03$ mol·L^{-1} after 96 h when the growth curve had reached its maximum within the late bacterial exponential phase. The results showed that the strain had a promising ability to degrade TCA by producing dehalogenase enzyme when cell-free extracts were prepared from growth on TCA as the sole carbon source with enzyme-specific activity, 1.1 ± 0.05 μmolCl^{-}min^{-1}·mg^{-1} protein. Furthermore, the morphological, and biochemical aspects of the isolated bacterium were studied to identify and characterize the strain. The morphological observation of the isolated bacterium was seen to be a rod-shaped, Gram-positive, motile, heterotrophic, and spore-forming bacterium. The amplification of the 16S rRNA and gene analysis results indicated that the isolated bacterium had 98% similarity to *Lysinibacillus boronitolerans*. The morphological and biochemical tests supported the 16S rRNA gene amplification. To the best of the authors' knowledge, this is the first reported case of this genus of bacteria to degrade this type of halogenated compound.

Keywords: biodegradation; dehalogenase-producing bacteria; haloalkanoic acids; trichloroacetic acid; 16S rRNA; *Lysinibacillus*

1. Introduction

Xenobiotic compounds are synthetic molecules which include halogenated hydrocarbon, polyaromatic hydrocarbons, polycyclic biphenyls, and lignin [1,2]. They are biologically active compounds that are widely used in several drug and pesticide industries [3,4]. Hence, xenobiotic compounds often appear in industrial wastewaters and aquatic ecosystems [5,6]. Xenobiotic compounds can be considered stable, in the thermodynamic sense. Moreover, they are fairly resistant to biodegradation by the native microorganisms, and persistent in the environment [7]. Damage caused by xenobiotic compounds poses significant health and ecological risks in developing countries [8]. The parent xenobiotic compounds are not directly toxic but they can be transformed into harmful oxy-radicals or carbon-centered radicals that attack the double bonds of cellular macromolecules generating oxidative damage [9]. Xenobiotic compounds have the potential to cause toxic effects on humans and animals with consequent acute carcinogenic, teratogenicity, and mutagenic effects [7]. Nonetheless, a diverse group of aerobic and anaerobic groups of bacteria often found in various habitats are capable of degrading xenobiotic compounds [10]. Several researches exist about biodegradation of xenobiotic compounds using dehalogenase-producing bacteria such as organofluorine [11,12], organochlorine [13,14], and organobromine [15,16].

Halogenated hydrocarbons are organic compounds that have many significant industrial applications such as multipurpose solvents, plasticizers, pesticides, fuel additives, flame retardants, and anesthetics [17–19]. Several halogenated hydrocarbon like polychlorinated biphenyl (PCB) residues and chlorinated hydrocarbon pesticide residues were detected in human adipose tissue, milk, and blood serum. Transfer and accumulation of these chemicals in the human body will consequently cause serious health problems [20]. One of the most well-known applications of halogenated aliphatics in the industry is related to the mixed substituted chlorofluorocarbons, CFC [21]. Millions of tons of halogenated aliphatics are produced annually and mainly have been used in a variety of manufacturing processes of solvents and cleaning agents [22]. These chemical compounds are common pollutants of aquatic habitats, as they are relatively water-soluble and can migrate to underground water-supplies and therefore threaten groundwater quality [23].

Haloacetic acids (HAAs) are recognized as oxidation products of airborne C_2-halocarbons which have been demonstrated to be fast in volatilization [24]. They are carcinogenic in humans even at low concentrations [25]. Several studies show that haloacetic acids are biodegradable in anaerobic environments including soil and, wastewater, [26]. HAAs come either from brominated and/or chlorinated organic halogen compounds. These organic halogenated compounds are important by-products of the reaction between organic compounds present in water and chlorine in water-treatment plants [27]. There are five contaminating by-products of halogenated organic compounds, which exceed the maximum levels established by the US Environmental Protection Agency (60 $\mu g \cdot L^{-1}$). These by-products include monochloroacetic acid (MCA), dichloroacetic acid (DCA), trichloroacetic acid (TCA), monobromoacetic acid (MBA), and dibromoacetic acid (DBA). As a result, the literature reporting on the analysis of HAAs mostly is focused on these five species [28]. TCA is a member of a very small group of "moderately strong" acids with ionization constants in the range 0.1–10 that is the most commonly used agent for chemical peeling [29,30]. TCA is one of the main contaminants of the environment that are carcinogenic to humans and animals [31]. The DCA and TCA are the most abundant HAAs detected in water resources containing nearly 80% of existing HAAs [32]. Hence, the decontamination of water resources from these compound is one of the major challenges for the preservation of the aquatic ecosystem and wastewater treatment [33].

Biodegradation is one of the main natural processes for the removal of xenobiotics such as chloroaliphatic compounds from the environment using microorganisms [34,35]. The major chemical processes for the metabolism of xenobiotic compounds are oxygenation, oxidation, reduction, hydrolysis, and conjugation, which are catalyzed by enzymes [36]. Dehalogenases are known as enzymes that catalyze the degradation reaction of halogen atoms from halogenated organic compounds. Dehalogenase-producing bacteria can detoxify harmful halogenated compounds from their substrates

by cleaving carbon–halogen bonds that link halogens in aliphatic compounds [37,38]. Based on cleavage nature, dehalogenase enzymes can be classified as haloalkane, dichloromethane, halohydrin, and L- and D-haloalkanoic acid dehalogenases [39]. Each specific group of these enzymes has its enantioselectivity and product inhibition characteristics [40]. Dehalogenase enzyme is present in bacterial systems and plays an important role in the biodegradation of environmental pollution caused by halogenated hydrocarbons [41].

Several research projects have been conducted to study the degradation mechanism of dehalogenase-producing bacteria for bioremediation of halogenated hydrocarbons and related hazardous compounds in soil and water ecosystems. Muslem et al. [42] characterized *Bacillus cereus* WH2 strain for its ability to metabolize β-haloalkanoic acids as carbon and energy sources. Alrawas and Huyop [35] isolated *Ralstonia* sp. TCAA-2 native to Danga Bay coast, Malaysia, to biodegrade TCA. Hamid et al. [43] further characterized dehalogenase enzyme functions of *Rhizobium* sp. RC1 bacteria. It was reported that RC1 can grow in α-haloalkanoic acid as the sole carbon source. α-haloalkanoic acids are halogenated compounds that are widely liberated into the ecosystem through the use of weed herbicides in the agricultural sector. Adamu et al. [44] studied the catalytic properties and mechanistic analysis of the microbial dehalogenases specific in *Rhizobium* sp. RC1. The isolated strain was reported to have outstanding performance in detoxifying L-2-haloacid. Selvamani et al. [45] characterized deploying *Trichoderma asperellum* SD1 to degrade 3-chloropropionic acid in the terrestrial ecosystem. Bagherbaigi et al. [46] used *Arthrobacter* sp. S1 for degradation of α and β-haloalkanoic acids.

As such, most of the research to date has focused on bioremediation of aquatic and terrestrial habitats. Nonetheless, there is a very little study to decontaminate TCA using dehalogenase- producing bacteria and their focus is on terrestrial bacteria or the identified bacteria are non-native to the coastal region of Johor strait. To the best of the authors' knowledge, this is the first reported case that shows *Lysinibacillus* bacteria could degrade halogenated compounds such as TCA. Because of the issues described above, this study intends to characterize a native bacterium with the capability of degrading TCA. To this end, several bacterial strains capable of aerobically degrading TCA were isolated from the northern coastline of Johor Strait in Malaysia and evaluated for their morphological and biochemical characteristics to degradation capabilities of TCA. The phylogenetic tree of the isolated strain was constructed using 16S-rRNA sequencing and it was recognized that the characterized bacterium was a new subspecies of the genus *Lysinibacillus*. The new bacterium was given the scientific name of *Lysinibacillus boronitolerans* MH2.

2. Materials and Methods

2.1. Sampling, Enrichments, and Growth

The seawater sample was collected from the northern coastline of Johor Strait in Malaysia. The water sample (1 mL) was added into 250 mL flasks containing 100 mL of minimal medium and TCA as a carbon source. Minimal medium contained basal salts solution, trace metals solution; and distilled water. The composition and proportion of the constituent of the minimal medium are shown in Table 1. The medium was adjusted to pH 7 before autoclaving. Then, the bacterial culture was incubated aerobically at 30 °C conditions in an orbital shaker set at 200 rpm. In order to prepare a solidified medium, two flasks (labeled Flask 1 and Flask 2) were prepared. Flask 1 contained 1.6 g oxide agar added into 48 mL distilled water (DW). Flask 2 was filled with 10 mL of trace metal salts and 10 mL of basal salts added into 30 mL DW. Flask 1 and flask 2 were autoclaved separately at 121 °C, for 15 min at 101.3 kPa. After autoclaving, solution in both flasks 1 and 2 were mixed and 1 mL of 1 M concentration TCA was added to the mixture, and the obtained medium was poured on agar plates. After the solidification of the agar, bacterial dilution was streaked onto the plates. Among the isolated strains, three different bacterial colonies were detected that the colony with the highest growth rate was retained for further purification. No further study was carried out for the identification of the two other isolated bacteria strains. The selected colony was picked and sub-cultured by repeated

streaking on a fresh agar plate to purify for further analysis. The selected colony was diluted in a drop of distilled water and transferred into minimal medium containing the ingredients shown in Table 1.

Table 1. Composition and proportion of the constituents in trichloroacetic acid (TCA)-enriched minimal medium.

Composition	Proportion	Composition	Name	Formula	Reference
Basal salts	10 mL	42.5 g·L^{-1}	Dipotassium hydrogen phosphate 3-hydrate	$K_2HPO_4{\cdot}3H_2O$	Hareland et al. [47]
		10 g·L^{-1}	Sodium dihydrogen phosphate 2-hydrate	$NaH_2PO_4{\cdot}2(H_2O)$	
		25 g·L^{-1}	Ammonium sulfate	$(NH_4)_2\,SO_4$	
		1.0 g·L^{-1}	Nitrilotriacetic acid	$N(CH_2COOH)_3$	
		2.0 g·L^{-1}	Magnesium sulphate	$MgSO_4$	
Trace metals solution	10 mL	0.12 g·L^{-1}	Iron sulphateheptahydrate	$FeSO_4{\cdot}7H_2O$	Hareland et al. [47]
		0.03 g·L^{-1}	Magan sulfate heptahydrate	$MnSO_4{\cdot}7H_2O$	
		0.03 g·L^{-1}	Zinc sulphate monohydrate	$ZnSO_4{\cdot}H_2O$	
		0.01 g·L^{-1}	Cobalt chlorine hexahydrate	$CoCl_2{\cdot}6H_2O$	
TCA	1 mL	10 mm	Trichloroacetic acid	$C_2HCl_3O_2$	Fisher Scientific
Distilled water	79 mL	-	Distilled water	H_2O	-
Total volume	100 mL	-	-	-	-

The bacteria were grown in minimal medium containing TCA as the sole carbon source. Chlorine atoms are eliminated by hydrolytic dehalogenation from the compound [48]. Chloride ion liberation had been monitored by the halide ion assay to prove the degradation of TCA [49]. The concentration of chloride ions was determined by converting absorbance value to concentration in mmol·L^{-1} based on the standard curve constructed using sodium chloride as the standard measurement of soluble chloride [50,51]. The suspension of the selected colony was used to evaluate the growth profile and TCA degradation. Several direct and indirect methods can be used for the evaluation of the bacterial growth profile. A colony-forming unit (CFU) is a direct method to evaluate the actual colony forming units per volume of culture in given bacterial incubation. The enumeration of CFU is one of the most accurate methods to find out the number of viable bacteria strains growing in the presence of harsh chemicals [52]. On the other hand, the spectrophotometric method is a widely used laboratory technique to indirectly estimate the bacterial concentration and quantitative analysis of the released chloride ions [44,53]. Spectrophotometry methods are based on the absorption and scattering of light beams passing through the culture medium. These methods are sensitive and selective; however, enumeration of CFU by plate count might be a disadvantage due to the fact that cells grown in clusters into a single colony and assumption that each colony arises from one cell is not accurate. In addition, commercial TCA may contain impurities and colony count may due to the growth of impurities rather than on TCA. In this study, absorbance A_{680nm} and A_{460nm} are used for analysis of the bacterial growth and released chloride ions, respectively. Chloride was liberated during the growth of TCA. The absorbance of the released chloride ions was recorded every 6 h using Jenaway spectrophotometer at A_{460nm}. Growth experiment and chloride ion released analysis were carried out in triplicate.

2.2. Preparation of Cell-Free Extracts and Dehalogenase Enzyme Assay

Apart from measuring cell growth (A_{680nm}) and chloride ions released (A_{460nm}) in the growth medium, the presence of dehalogenase was assessed as the liberation of halide in cell-free extracts in vitro. Cells from a 100 mL culture grown in 10 mm TCA and nutrient broth were harvested by centrifugation at 20,000× *g* for 10 min at 4 °C during the late-growth phase, between 72–96 h (Figure 1). The pellets were resuspended in 10 mL of 0.1 M tris-acetate, 1 mm ethylenediaminetetraacetic acid (EDTA), 10% (mass·vol^{-1}.) glycerol, pH 7.5, and washed three times with 0.1 M tris-acetate buffer by centrifugation at 20,000× *g* for 10 min at 4 °C. Finally, the cells were then resuspended in 3 mL of the same buffer and maintained at 0 °C for sonication in an MSE Soniprep 150 W ultrasonic disintegrator at

a peak amplitude (λ = 10 microns) for 30 s. Any unbroken cells and cell wall materials were removed by centrifugation at 25,000× *g* for 20 min at 4 °C. The cell-free extracts activity was assayed immediately after the preparation as previously described by Mesri et al. [54].

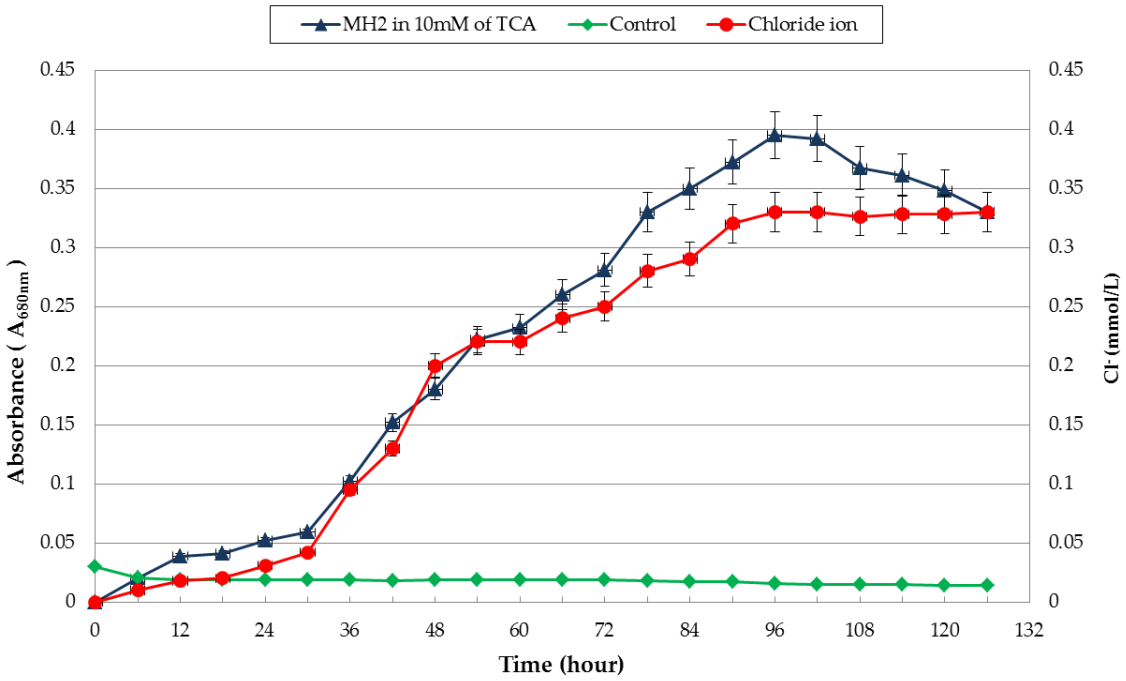

Figure 1. The growth curve of the MH2 bacterium measured at A_{680nm} and chloride ions released measured at A_{460nm}. The control culture without strain MH2 was incubated under the same growth conditions. All readings were based on triplicate analysis.

2.3. Biochemical and Morphological Characterization

Morphological tests, namely Gram staining, spore staining, and motility tests as well as biochemical tests including catalase, oxidase, urease, gelatin liquefaction, citrate, MacConkey agar, casein hydrolysis, starch, indole, and triple sugar iron (TSI) tests, were carried out for basic identification of bacteria generics and inferring their properties following the standard outline in Bergey's manual [55]. Table 2 shows the morphological and biochemical characteristics of the selected bacteria.

Table 2. Morphological and biochemical characterization of *L. boronitolerans* MH2.

Objective	Test	Composition and Materials	Incubation Condition	Aim	Description	Reference
Characterization of bacteria	Gram staining	• Crystal violet • Iodine • Alcohol • Safranin	-	To determine the type of bacteria (Gram-positive or gram-negative)	Smear color	[55]
	Spore staining	• Safranin • Malachite	-	To determine the spore production of bacterium	Retaining cell color	
	Motility	• 5.0 g peptone, • 2.0 g agar • 2.5 g NaCl • 1.5 g beef extract • 200 mL distilled water (DW)	Incubated at 30–37 °C for 24–48 h.	To determine the motility and to explore the capability of bacteria to migrate away from a line of inoculation	Turbidity diffusion from the stab line	
Biochemical tests	Catalase	• Hydrogen peroxide	-	To differentiate aerobic and obligate anaerobic bacteria	Bubble formation	[56,57]
	Oxidase	• N′N′N′N′-tetramethyl-p -phenylenediamine36dihydrochloride (TMPD)	-	To determine the taxonomic status and identity of pathogenic bacteria	Color change	
	Urease	• 5.5 g of Christen′s urea agar • 50 mL of 40% urea solution • 250 mL DW	Autoclaved at 121 °C for 20 min. Incubated at 37 °C 24–48 h.	To determine the ability of bacteria to decompose urea which produces the enzyme urease	Color change	
	Gelatin Liquefaction	• 0.75 g of beef extract • 1.25 g of peptone • 30 g of gelatin in • 250 mL DW	Incubated at 33 °C for four days	To explore the ability of an organism to produce extracellular proteolytic enzyme	Solidification and hydrolysis of gelatin	

Table 2. *Cont.*

Objective	Test	Composition and Materials	Incubation Condition	Aim	Description	Reference
Biochemical tests	Citrate	• 3.5 g of Simmon's citrate in • 150 mL DW	Autoclaved at 121 °C for 20 min. Incubated at 37 °C for 24–48 h.	To look at the ability of enteric organisms based on the ability to ferment citrate as a carbon source	Color change	[56,57]
	MacConkey Agar	• 17 g peptone • 3 g proteose peptone • 10 g lactose monohydrate • 1.5 g bile salts • 5 g sodium chloride • 0.03 g neutral red • 0.001 g crystal violet • 13.5 g agar • DW (to adjust 1 liter)	Autoclaved at 121 °C for 20 min. Incubated at 37 °C for 24–48 h.	To isolate Gram-negative enteric bacteria and the differentiation of lactose fermenting from lactose non- fermenting Gram-negative bacteria	Colony observation	
	Casein hydrolysis	• 3.6 g nutrient agar • 0.5 g casein • 200 mL DW	Incubated at 33 °C for three days	To identify bacteria capable of hydrolyzing casein with casease enzyme.	Observation of clear zone	
	Starch	• 1.5 g soluble starch • 2.5 g bacteriological agar • 150 mL DW	Autoclaved at 121 °C for 20 min. Incubated at 37 °C. for 24–48 h	To determine the amylase enzyme with the ability to degrade starch	Color formation	
	Indole	• 1 g sodium chloride • 2 g tryptophan or peptone • 200 mL DW	Autoclaved at 121 °C for 20 min at pH 7.5	Tryptophanase enzyme	Color formation	
	Triple Sugar Iron (TSI)	• 1.5 g soluble starch • 2.5 g bacteriological agar • 150 mL DW	Autoclaved at 121 °C for 20 min; incubate for 24–72 h	To test a microorganism's ability to ferment sugars and to produce acid	Color formation	

2.4. Molecular Identification

Genomic DNA from bacterial cells of strain MH2 was isolated using the Wizard® Genomic Purification Kit (Promega). Universal polymerase chain reaction (PCR) forward and reverse primers Fd1 (5′-aga gtt tga tcc tgg ctc ag-3′) and Rp1 (5′-acg gtc ata cct tgt tac gac tt-3′), respectively, were synthesized by 1st Base® Laboratory Malaysia Sdn. Bhd., 43300 Seri Kembangan, Selangor, Malaysia [58]. The amplified 16S rRNA gene has to be purified before sequencing. QIAquick polymerase chain reaction (PCR) purification kit (Qiagen) was used to purify the DNA. The sequencing was carried out by ABI PRISM® 377 DNA sequencer (1st Base® Laboratory Malaysia Sdn. Bhd., 43300 Seri Kembangan, Selangor, Malaysia). The thermocycling conditions for PCR amplification are presented in Table 3.

Table 3. Thermocycling conditions for polymerase chain reaction (PCR) amplification.

Step	Temperature	Time	Cycles
Initial Denaturation	94 °C	5 min	1
Denaturation	94 °C	1 min	
Annealing	55 °C	1 min	30
Elongation	72 °C	4 min	
Final Elongation	72 °C	10 min	1

The phylogram of unidentified bacteria was rebuilt using Mega5 Molecular software and compared with the sequences from the 16S rRNA gene stored in the Gene Bank by the National Center for Biotechnology (NCBI) using the Basic Local Alignment Search Tool (BLASTn) for nucleotides [59]. Sequences were aligned using Bioedit Sequence Alignment Editor X and a neighbor-joining tree was constructed [60]. Bootstrap consensus tree was inferred from 500 replicates.

3. Results

A seawater sample was taken from the marine environment and three strains of bacteria were isolated using minimal media. Once the bacterial strains were purified, only one isolate (MH2) was selected from three bacterial samples due to its distinctively superior growth and survival abilities in solid minimal media compared to the other two bacteria for further analysis. The doubling time for MH2 strain was found 32 h and the maximum released chloride ion due to dehalogenase enzyme activity was approximately $0.33 \times 10^{-3} \pm 0.03$ mol·L^{-1}. 16S rRNA phylogenetic analysis was conducted to determine the phylogenetic relatedness and the species affiliation of the MH2 strain.

3.1. Growth of L. boronitolerans MH2

The growth profile of the isolated *L. boronitolerans* MH2 strain bacteria was examined in fixed time intervals. The growth profile exhibiting the lag, exponential, stationary, and decline phase as it was expected. The growth of the bacteria by measuring through light absorbance of the solutions measured at 680 nm (A_{680nm}) by using a JENWAY, 6300 ultraviolet (UV)-visible spectrophotometer at every 6 h. The measured values for the growth of MH2 were recorded based on 10 mm of the TCA-enriched medium. In the lag phase, the bacterium growth was very little because the bacterium does not immediately adapt to the growth condition and the carbon source which was toxic to the bacteria. In the exponential phase, the growth rate of the MH2 bacterium increased. At the end of the exponential phase, the growth curve reached its maximum rate at 96 h. After a long exponential phase, the growth curve reached a stationary phase and then the bacterium enters the decline phase. The growth profile shows that MH2 was able to grow in minimal medium containing 10 mm of TCA. According to Slater et al. [51], the bacterium absorbs TCA from the compound as the sole carbon source and releases chloride ions. The liberated chloride ion during the metabolism of MH2 strain was monitored by a halide ion assay order as an indicator of TCA degradation in the medium [51].

The results of chloride ion released and the growth curve of the bacterium was plotted as shown in Figure 1.

Figure 1 shows that both the halide ion assay and growth curves of the bacterium in minimal medium exhibited identical trends indicating that the concentration of the released chloride ion and the growth are directly related to each other. The curves for halide ion assay and growth of MH2 were in contrast with the control experiment. The concentration of the chloride ion in the cultivation solution was determined by converting absorbance corresponding to the concentration using the standard curve. The standard curve was constructed using NaCl as a typical standard for measuring soluble chloride concentration [61]. Doubling time of the bacteria in 10 mm TCA-enriched minimal medium was estimated to be 32 h.

3.2. Enzyme Activity in Cell-Free Extracts from Cell Growth in Trichloroacetic Acid (TCA) Medium and Nutrient Broth

Dehalogenase activity in cell-free extracts was assessed by the release of free halide in an enzyme-substrate reaction in vitro. Cell-free extracts from strain MH2 grown on 10 mm TCA medium as the sole carbon source was prepared. Halide liberation was measured from extracts of cells grown in TCA using TCA as a substrate. However, no halide was liberated by extracts of the same bacteria grown in nutrient broth. This suggests that in nutrient broth no expression of the bacterial dehalogenase gene takes place. The enzyme specific activity of the dehalogenase for TCA was 1.1 ± 0.05 $\mu molCl^{-}min^{-1} \cdot mg^{-1}$ protein.

3.3. Morphology and Biochemical Characteristics

The result of the bacteria characterization based on morphological and biochemical analysis shows that the isolated bacterium grew well at aerobic conditions and can readily metabolize TCA and produce chloride ions by dechlorination treatment. The morphological observations for Gram-staining and spore staining using light microscopy 1000× magnification are shown in Figures 2 and 3, respectively. The result of Gram-staining analysis indicated that the MH2 strain is a Gram-positive bacterium and produced creamy to pink colonies on solid minimal medium. *L. boronitolerans* MH2 was further examined for biochemical characteristics.

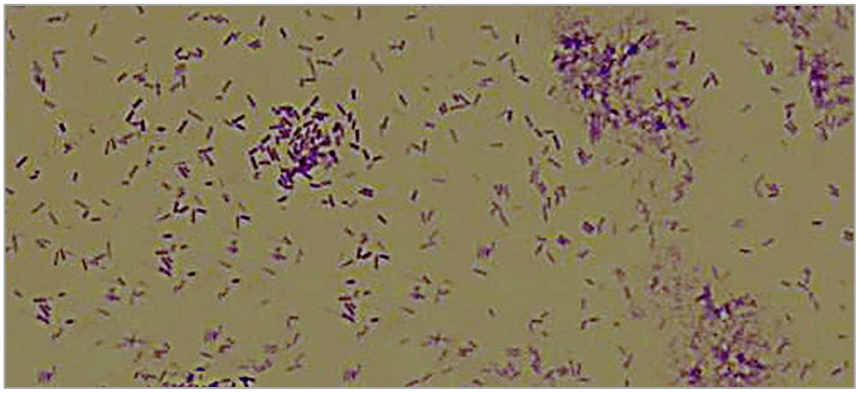

Figure 2. The Gram-staining of strain MH2 observation under light microscope (×1000 magnification). Purple colour bacteria appeared as the result of Gram staining which indicated MH2 was a Gram-positive bacterium.

A spore staining test was performed to determine if MH2 is an endospore-forming bacterium. Spores were observed during the microscopic examination that proves that bacterium MH2 was capable of producing spores. Microscopic observation of the spore staining is shown in Figure 3.

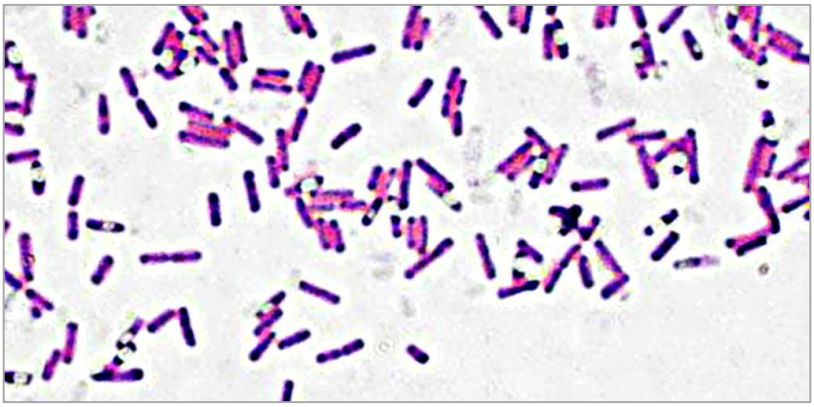

Figure 3. Spore staining for strain MH2 observed under a light microscope (×1000 magnification). Spores were seen as black spots in each cell.

Tables 4 and 5 showed the summary of morphological characteristics of MH2 and the results of biochemical tests, respectively. The oxidase, urease, casein, and starch hydrolysis tests showed positive results and catalase, gelatin, citrate, MacConkey agar, indole, and TSI demonstrated negative results. Morphological and biochemical tests were conducted based on Bergey's manual systematic bacteriology [56].

Table 4. Morphological character of MH2.

Characteristic	Parameter
Identity	*L. boronitolerans* MH2
Cell shape	Rod shape
Size	0.5~1 mm
Colony	Cream
O_2 requirement	Aerobic
Colony morphology	Smooth, mucoid, a little elevated

Table 5. Morphological and biochemical characteristics of MH2.

Test	Spore Forming	Gram Staining	Motility	Catalase	Oxidase	Urease	Gelatin	Citrate	MacConkey Agar	Casein Hydrolysis	Starch	Indole	TSI
Result	+	+	+	−	+	+	−	−	−	+	+	−	−

Positive (+); Negative (−).

3.4. Polymerase Chain Reaction (PCR) Amplification of 16s rRNA Gene Analysis

Colony PCR (cPCR) is performed to verify the construct of the DNA-sequence. 16S rRNA genes were amplified using universal primers Fd1 and Rp1, respectively. The amplified genes were monitored with agarose gel electrophoresis. In Figure 4, the unique amplified band (1500 bp) was observed and compared with the 1 Kb DNA ladder.

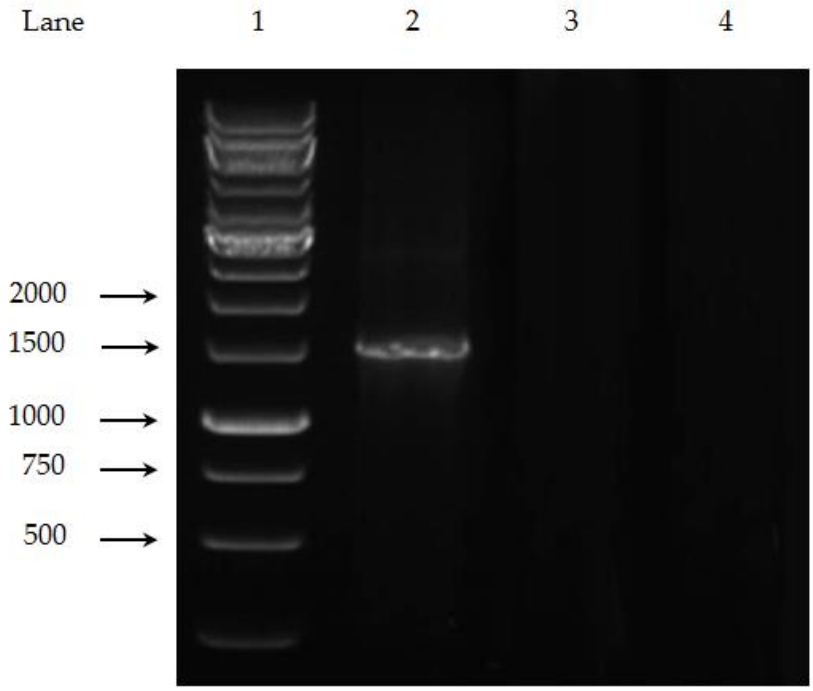

Figure 4. Amplification of the 16S rRNA gene of strain MH2 showing 1500 bp DNA fragment on an agarose gel (1%) (Lane 2). Lane 1: 1kb DNA ladder (Promega); Lanes 3 and 4, negative controls, polymerase chain reaction (PCR) mixture without forward (Fd1) and reverse (Rp1) primer respectively, showing no amplification.

The continuous stretch (1274 bp) of 16S rRNA gene was investigated to determine the closest phylogenetic neighbors. The BLAST program was employed and it was revealed that the MH2 has 98% identity matches in sequence with *L. boronitolerans*. Amplification and direct sequence analysis of partial length of 16S rRNA indicate that bacterium MH2 is phylogenetically related to *L. boronitolerans*. The results of the 16S rRNA analysis supported the biochemical characteristics of the bacterium belong to the same genus and species.

3.5. Phylogeny Tree Analysis

The phylogenetic tree was constructed using the neighbor-joining method and the maximum composite likelihood method. The bootstrap consensus tree offered from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are excluded. The percentage of replicate trees is shown next to the branches. Evolutionary analyses were conducted in MEGA5 [59]. Figure 5 presented the phylogeny tree of the MH2 strain.

The maximum composite likelihood method was used to estimate the evolutionary distances. The units of evolutionary distances were based on the number of base substitutions per site; 11 nucleotide sequences were involved in the analysis. All gaps and missing data were excluded for tree building before analysis. Only 1238 positions were considered in the final dataset.

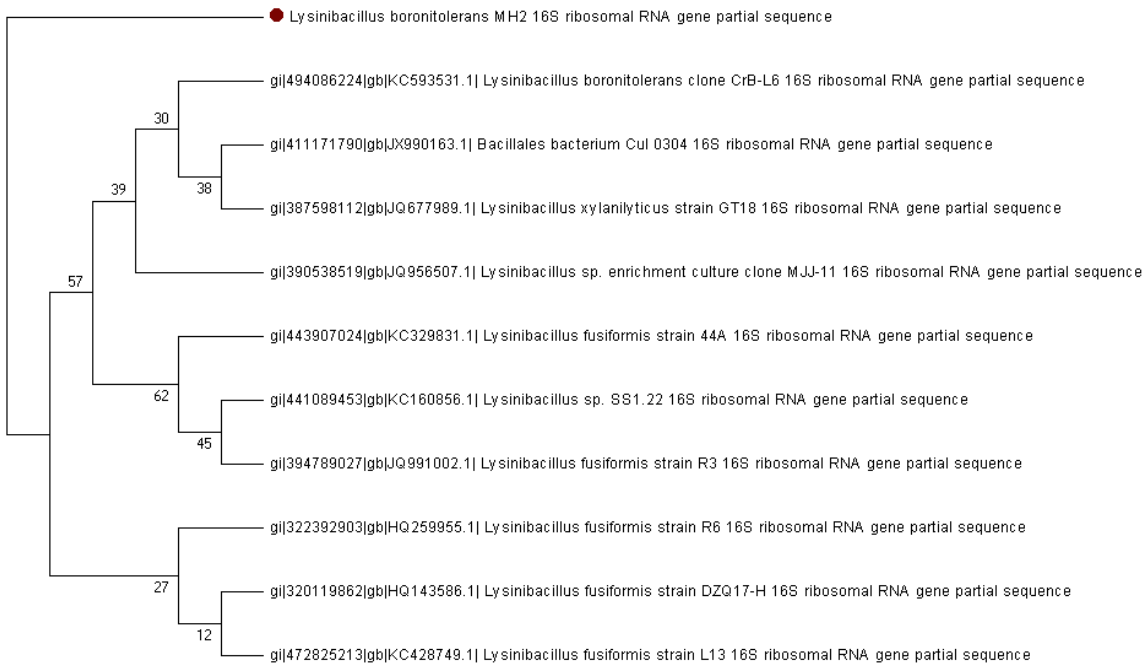

Figure 5. Neighbor-joining tree of strains M2 based on 16S rRNA gene sequences. Scale Bar 0.001 indicates sequence divergence.

4. Discussion

Biodegradation is an effective biological process to clean up polluted environments through the isolation of efficacious halo-organic compound biodegraders. Notably, the BLASTn search on the 16S rRNA gene sequence revealed that MH2 has the 98% identity match with *Lysinibacillus boronitolerans* (accession number: KC59351.1). Hence, to identify the capability of MH2 to degrade the sample pollutants, this study grew the MH2 bacterial isolate in TCA-enriched minimal media as the sole carbon source (pollutants).

The initial composition of the culture medium and the concentration of the substrate (TCA) are two important parameters that can affect the degrading ability of the bacteria. Using artificial seawater or sterilized seawater as a medium for cultivation of sea-isolated bacterium was refrained from due to the potential interference of natural chloride ions in seawater with those liberated during the growth experiment. Minimal medium contained basal salts solution, trace metals solution, and distilled water was used in this study to provide the required growth culture for the bacteria. The existing nitrilotriacetic acid ($N(CH_2COOH)_3$) in the trace metals solution is low enough not to interfere significantly with the results of the experiment and to be consistent with the hypothesis of utilizing TCA as the sole carbon and energy source in the bacteria growth process. The optimal concentration of the TCA pollutant sample was selected 10 mm in the study of the growth profile and the ion liberation experiments. Using TCA in concentrations higher than 20 mm can stifle the cell growth due to toxic effects while the lower concentration of pollutants was not enough to observe the induced catalytic reaction of the dehalogenase-producing bacteria. Growth was strictly monitored by measuring the cells' turbidity and the amount of chloride ions released at appropriate time intervals. An uninoculated flask treated in the same way was used as a control. This is important to make sure the chloride measured in the growth medium was due to the cells using the TCA rather than the auto-degradation of the substrate in the growth medium.

MH2 strain was capable of degrading hydrocarbon compounds. The nature and type of carbon sources are among the most important factor to determine bacterial growth. Haloacetates (i.e., MCA, DCA, and TCA) are common classes of water chlorination by-products. Several bacteria are available that can grow on MCA. *Burkholderia* sp. DehCL1 [62], *Bacillus* sp. TW1 [63], *Xantobacter autotrophicus*

GJ10 [64,65], and *Pseudomonas* sp. R1 [66] are examples of bacteria that can degrade MCA haloacetates. Additionally, Meusel and Rehm [67] described *Xanthobacter autotrophicus* GJ10 that can degrade DCA. To the best of the authors' knowledge, this is the first report on *Lysinibacillus* sp. isolated from seawater adapted in metabolizing TCA.

The results showed that the highest growth in *L. boronitolerans* MH2 bacterium was achieved at 96 h. Low absorbance was observed for the lag phase due to the low intensity and slow growth of the bacterial biomass produced. In broth, the doubling time of the *L. boronitolerans* MH2 was 32 h. Chloride ion concentration during the biodegradation process was monitored by a chloride ion assay [61,68]. Based on the standard curve of the NaCl, the released chloride ion by *L. boronitolerans* MH2 strain after 96 h was 0.33×10^{-3} mol·L^{-1}. The study of the growth curve shows that the *L. boronitolerans* MH2 was capable of growing in a minimal medium having TCA as the sole source of carbon and energy. Growth on TCA was further analyzed by preparing the cell-free extracts from growth on TCA as described in Figure 1 and Section 3.2. The presence of dehalogenase was detected by measuring the enzyme-specific activity. The *L. boronitolerans* MH2 dehalogenase described herein appears to be inducible because MH2 cells grown in nutrient broth lacking TCA exhibited no dehalogenase activity.

Hydrolytic dehalogenation of TCA produces oxalate as a final product where oxalate serves as the carbon source and it can be immediately converted to CO_2 [69]. The number of bacteria capable of using oxalate as the sole source of carbon is very limited [70]. Biochemical and morphological experiments were carried out according to Bergey's manual [56] aimed at verifying the obtained result of 16S rRNA gene analysis. The results of biochemical characteristics supported the findings of the bacteria suggested by the 16S rRNA analysis. The growth profile of the isolated strain was examined and the results showed that *L. boronitolerans* MH2 bacterium has a promising ability as a dehalogenase-producing bacterium.

5. Conclusions

Using dehalogenase enzymes to detoxify chlorinated organic compounds was envisaged as a promising biological control method. The applicability of *L. boronitolerans* MH2 is an important outcome discovered in current analysis suggesting dehalogenase-producing bacteria is important for the future to the exploitation of the bacterium for in situ efforts to detoxify halogen-contaminated environments. More importantly, these findings further add to the limited body of knowledge with regards to the degrading of halogenated compounds by the bacteria.

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