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Original Article

Isolation and identification of bacteria degrading 2,2-dichloropropionic acid in water sample

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Abstract: The widespread use of herbicides containing 2,2-dichloropropionic acid (2,2-DCP) as a recalcitrant halogen compound poses significant environmental risks and can be harmful for human. Consequently, it is important that the bio-based detoxification method is developed in an environmental manner. This study was aimed to isolate and identify a possible degradation 2,2-DCP bacterial strain as the sole source of carbon. A bacterial dehalogenase producing 2,2-DCP was isolated named as WM. The WM strain was shown to have 98% sequence identity and characteristics similar to *Enterobacter* sp. based on 16s rRNA analysis, biochemical and morphological tests. Phylogenic analysis showed that the WM strain is *Enterobacter* sp.. In media with 20 mM 3CP, the bacteria were well growing at 37°C, although an optimal chloride ion release was 0.48 μmol Cl/mL. Our finding is first report of an *Enterobacter* sp. strain which can use 2, 2-DCP as sole carbon source in a competent manner.

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Introduction

Our environments specially in aquatic ecosystems are seriously affected by increased levels of industrial chemicals, wastes and biocides. This is led important research programs to examine the concentraion of these substances in the nature (Ejlertsson et al., 1996; Baumann et al., 2005). Classifications of chemical compounds in the environment are based on natural and biosphere-free products according to the groups (Hutzinger and Veerkamp, 1981), which have not natural origin but from human activities defined as xenobiotics. Temporary or permanent xenobiotic contamination may affect biosphere's normal functions. Modern agriculture lead to applications of herbicides and pesticides (Atlas, 1996). In addition, numerous routes could introduce pollutants in the environment such as illegal dumping, leaked effluents, liquidation and chemical evaporation. The full extent of harm to the environment should be evaluated by effects such as toxicity, carcinogenicity, bio-agrandissement and persistence.

Halo-aliphatic compounds such as 2,2-dichloropropionic acid and trichloroacetic acid are common herbicides (Edbeib et al., 2020). 2,4-dichloroxyacetate (2,4-D) and 2,4,5-trichloro-tri-chlorophenoxyacetate (2,4,5-T) are of great interest for their degradation and therefore many researchers were initiated to investigate their environmental fate (Ghosal et al., 1985; Farhana et al., 1987). Halo-organic compounds (e.g. methoxychloride, Lindane, Aldrin and 1,1,1 trichloro-2,2-bis (DDT)) contributed to the contamination of the environment (Ritter et al., 1995). However, naturally occurring halogenated compounds are usual. In soil and fresh water ecosystems, halogens such as inorganic salts or minerals are relatively abundant (Fetzner, 1998). More than 2000 halo-organic compounds are released by various marine organisms, plants, fern, insects, bacteria and fungi into the biosphere as natural products (Gribble, 1994). Furthermore, analogs of

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bromins and iodine chloralkans in natural conditions have been identified (Gschwend et al., 1985) and can be seen as biodegradation models for more halogenated alkanes, in view of their chemical and analytical implication.

The primary agents of biological recycling are microorganisms, which have evolved a broad range of enzymes, pathways and control mechanisms to degrade and use of the pollutants as energy sources (Madigan et al., 2000; Talaro and Talaro, 2002). There is no evidence from laboratory studies shows the involvement of microorganisms in transforming halogenated xenobiotics as an important factor in determining their environmental fate (Talaro and Talaro, 2002; Muslem et al., 2018). As many other organisms participates, but to a lesser extent, microorganisms are not exclusively responsible for degradation of organic compounds. Photochemical decomposition was proposed as a major way of degrading certain compounds (Zabik et al., 1976). All existing organic compounds are considered thermodynamically unstable in varying degrees, and CO_2 and microbial growth energy can be generated in principle. But most organic compounds are completely stable in the kinetic sense, and they are not degraded or mineralized at significant rates under physiological conditions without catalysis. Such enzyme-based catalysis is common in aerobic microorganisms seeking growth energy. It is a difficult chemical and biological activity relationships and may help to the recalcitrance of some halogenated compounds. The removal, especially from organic molecules of halogens, fluorines and chlorine has long been fascinating for chemical and microbiology professionals because these mechanisms ease the inhibitory effect and provide alternative carbon and energy sources for growth (Slater et al., 1995).

In general, the number of halogens per organic molecule is more difficult to degrade alone or microbially (Commandeur and Parsons, 1990). Halogenated compounds are ideal pesticides. Leasure (1964) observed that chlorinated substrates are the most suitable herbicidal activity over brominated, fluorinated and iodinated compounds. But an α-chlorine result in the highest herbicidal activity in propionic acid and homologous butyric acid; DCA (dichloroacetate) and trichloromethane (MCA) do not result in activity, and with α-chlorinated materials, the proper effect can be weakened. Also, the increase in the chain length seems a weakly active 2,2-dichloropentanoic and 2,2-dichlorohexanoic inactive acid to decrease the activity even of very biocidal compounds.

The acidic properties of halogen when connected with the parent molecule may be another factor that can make haloorgan compounds desirable for biocidal action. Acid dissociation constant shows the relative strength of an acid (Ka). The stronger the acid, the greater the dissociation; logarithmically, Ka is often expressed because of a broad range of spans. Stronger acids would therefore have lower pKa values (Solomons, 1994; Wade, 2003). The more highly replaced aliphatic and aromatic compounds, the more strongly electron-negative. The acidic nature of the halo-organ compounds is also affected by the position of substitution, and the more the halogen replacement is separated from the –COOH group, the less acid is indicated by the pKa values, known as "inductive effect" phenomenon. The inductive effect is obvious when one or more groups strongly draw electron from the carbon. The extent of the substitution effect is determined by its distance from carboxylic acid, while distant substitution agents have less acidity and a rapid decline in distance induction effects (Wade, 2003).

Halogenized compounds may be used in crops as an herbicide to excessively damage the human and natural environment because of the persistent, biologically accumulative and toxic halogenated compositions. Such biocides are used for plant protection directly and deliberately in the environment. Underground water sources as well as plants could pollute by these non-degraded synthetic chemicals. Halogenated analogs of intermediate metabolites were also shown to be toxic because they inhibited a major reaction in organisms' central metabolism (Slater et al., 1995). For certain important pollutants, dehalogenation is the only known biodegradation system. It is usually less toxic and

easier to degrade xenobiotic compounds. The significance of the study has been derived from little available information regarding the bacteria isolates degrading 2,2-DCP compounds. Therefore, this study was aimed the isolation and identification of a possible degradation 2,2-DCP bacterial strain as the sole source of carbon.

Materials and Methods

Isolation of bacteria strain: In an agriculture area that haloacids have been utilized i.e. previously exposure to pesticide, a soil sample were removed and packed into sterile jars, sealed, and stored at 4°C.

Sample processing and purification: In 30 mL of the sterile distilled water, a 10 g soil sample is suspended, and the mixing was continued until the particles' settlement in the base. Then the pipettes are spread over plates with 20 mM of 2,2-DCP on 0.1 mL of the mix of suspension soil. The plate was incubated at 37°C for 6 days so that bacterial growth is permitted. One plate is incubated as a control along with the other with blank inoculation. After the incubation, the colonies are carefully isolated and streamed through the streak-plating method into the same type of minimal medium plate. The method of streak plate is continued to achieve a pure colony.

Media for growth: Based on Miller (1972), the yeast extract containing NaCl (10 g/l) and tryptone (5.0 g/l) was used as Luria Broth (LB) media. The 10x basal salts were prepared using $NaH_2PO_4.2H_2O$ (10.0 g/l), $K_2HPO_4.3H_2O$ (42.5 g/l) and $NH_4.2SO_4$ (25.0 g/l) for minimal concentrations of PJC chloride-free. The solution for trace metal salts were included 10x of $FeSO_4.7H_2O$ (120.0 mg/l), $MgSO_4$ (2.0 g/l), $MnSO_4.4H_2O$ (30.0 mg/l) and nitriloacetic acid ($C_6H_9NO_6$) (1.0 g/l) as well as disposed water $CoCl_2.6H_2O$ (10 mg/l) and $ZnSO_4.H_2O$ (30 mg/l) (Hareland et al., 1975). Minimal media for bacterial growth has been autoclaved (121°C for 15 min, 15 psi) containing 10 ml basal salts per 100 ml distilled water and 10 ml trace metal salts. Sources of carbon i.e. 1M 2,2-DCP were separately sterilized, then added to the desired final concentration aseptically to the media. 0.05% (w/v) final concentration was added to the liquid minimal cultures by yeast extract. Bacteriological oxoid agar (1.5% w/v) was added before sterilization to prepare a solid medium. Organism culture was prepared by adding 0.3 mL 50% sterile glycerol to 0.7 mL culture and then mixed with the sample thoroughly and frozen in dry ice. Cultivation of stocks was stored at -80°C.

Microbial growth measurement: The absorption was measured at 680 nm for microbial growth (A680 nm). For this, a sample of 2 ml culture was pipetted and absorption was measured in 680 nm in a spectrophotometer (T60 UV/VIS). Absorption readings have been taken at appropriate intervals for broth medium turbidity. A graph of log A680 nm against exponential phase time was determined to measure specific growth rate. μ was determined from the slope of the graph (Stanbury and Whitaker, 1984) and td equation time was calculated to double = 0.301/μ (Shuler and Kargi, 2002). he bacteria cultures were incubated using temperature-adjustable rotary shaker at 30±1°C, T150 rpm. Test media optical density (OD) was measured using A600 nm at every 6 hrs.

Growth profile: The WM isolate was first cultured in 20 mM of 2,2-DCP, and the culture was transferred to the test media. The test media were prepared in triplicates of liquid minimal media that consisted of three different concentrations of 2,2-DCP (10, 20 and 30 mM) and were based on substrate concentration and culturing condition as suggested (Mesri et al., 2009).

PCR and Sequencing: Following the PCR, the products were sent to the 1st Base® Company for DNA sequencing in both direct and reverse directions. Then, BioEdit software was used to observe the sequence. Finally, BLAST was used to find the similar bacteria species based on 16S rRNA gene (https://blast.ncbi.nlm.nih.gov).

16S rRNA gene phylogenetic analysis: The obtained 16S rRNA sequences were compared using the BLAST with other sequences (Table 1). The first ten similar sequences were retrieved and aligned using the CULSTALW. The phylogenetic tree was reconstructed with MEGA (Version V6) based on neighbor-

Table 1. The retrieved sequences with them accession number from GenBank.

Accession number	Description	max. score	total score	query coverage	max. identity
EU139848	*Enterobacter* sp. GJ1-11	2518	2518	98%	%99
EF059865	*Enterobacter cloacae* strain E717	2529	2529	98%	99%
KF478236	*Enterobacter cloacae* strain IPRI	2525	2525	98%	99%
HE978272	*Enterobacter cloacae* subsp.	2523	2523	98%	99%
NR028912	*Enterobacter cloacae* strain 279-56	2525	2525	98%	99%
FJ888607	*Enterobacter* sp. BSRA3	2523	2523	98%	99%
KF523893	*Enterobacter cloacae* strain GW-3	2521	2521	98%	99%
KF984470	*Enterobacter* sp. BAB-3361	2518	2518	98%	99%
JN986806	*Enterobacter* sp. Wy2-D9	2518	2518	98%	99%
AB644526	*Enterobacter cloacae* strain 56_2_1	2518	2518	98%	99%

joining algorithm (Saitou and Nei, 1987).

Halide ion assays: During the dehalogenation reaction, the free halides were measured by the adapting the Bergman and Sanik method (Bergmann and Sanik, 1957). Sample (1 ml) was added and carefully mixed in 9 M nitric acid (reagent A) to 100 µl of 0.25 M ferric sulphate ammonium. To this, 100 µl of mercuric ethanol saturated with thiocyanates was added (reagent B), and the solution mixed by vortex. The color was measured at A460 nm for 10 min. By comparing the absorbance of the samples to a standard curve of known halide concentrations, the halide concentration was determined. The standard halide ion assay curve was built on the basis of the chloride concentration of sodium chloride (NaCl) 0.01 M (10 mM). In each 0.8 mL of standard solutions in a clean cup, 0.1 mL of reagent A and 0.1 mL of reagent B were added and left for 10 min for color development in the cup (orange). The mix was measured at A460 nm. Approximately 1.5 mL of culture sample liquid was pipetted and centrifuged for cell removal at 10.000×g for determining the production of chloride by bacteria. 0.8 mL of the supernatant was transferred to a clean cup and A reagent of 0.1 mL plus 0.1 mL of the reagent B, then left to fill for 10 min. Finally, its absorption was read at A460 nm.

Results and Discussions

Isolation and characterization of 2,2-DCP degrading bacteria: The bacterial colonies were grown on a 20 mM 2,2-DCP minimal medium as the only carbon source 6 days after its culture (Fig. 1). One type of bacterial colony was identified as degrading bacteria after a series of sub-cultures of randomly selected primary plating colonies. This sole carbon source user at 37°C was named as "WM strain" that grown on a minimum medium of 20 mM 2,2-DCP. Analysis of bacteria's cellular morphology under a light microscope showed isolates of 20 mM 2,2-DCP minimum media agar which were usually formed in circular, white (pale brown through suntans) and creamy colonies. Gram staining showed that this strain i.e. WM, is a Gram negative one.

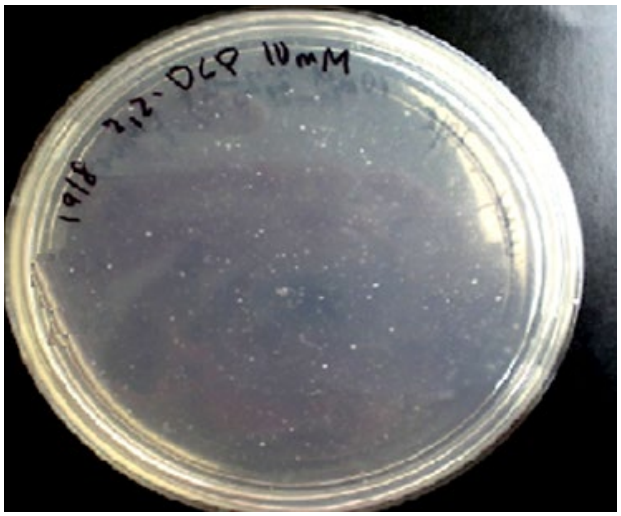
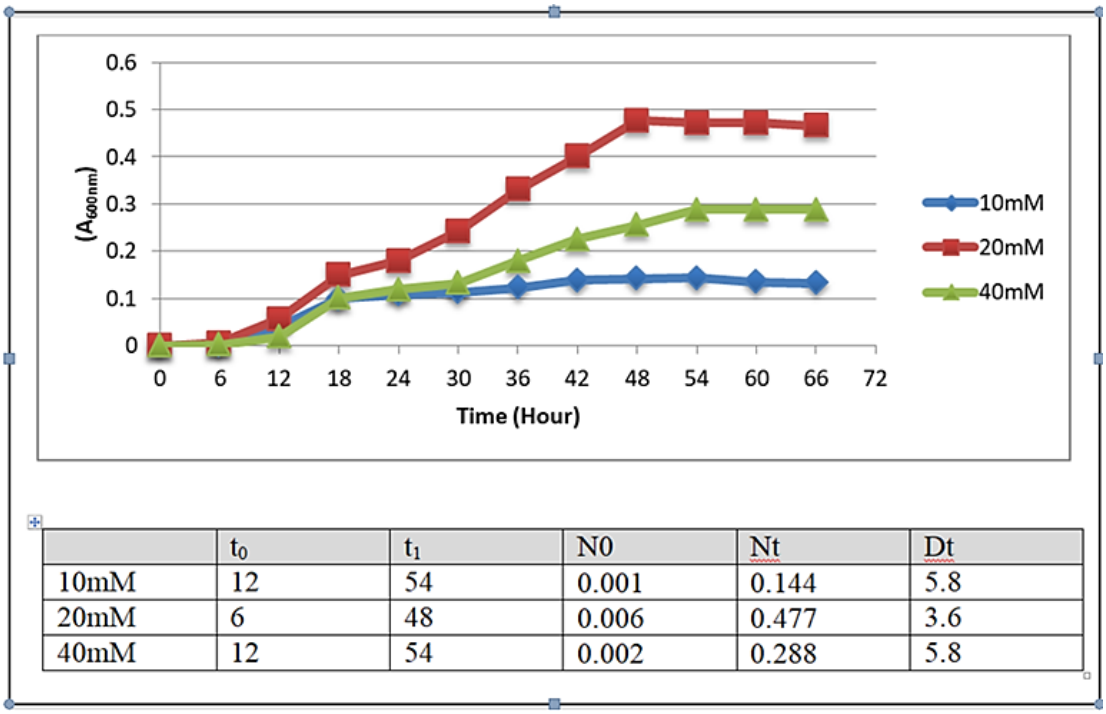


Figure 1. Growth on spread plate minimal media contain 20 mM 2,2-DCP observed after 6 days (incubated at 37°C).

Minimum liquid medium growth different 2,2-DCP concentration supplemented: The WM isolate growth was monitored as the sole source of carbon in liquid minimum media with differing concentrations



	t_0	t_1	N0	Nt	Dt
10mM	12	54	0.001	0.144	5.8
20mM	6	48	0.006	0.477	3.6
40mM	12	54	0.002	0.288	5.8

Figure 2. WM strain growth profile on minimum media of three different 2,2 DCP concentrations.

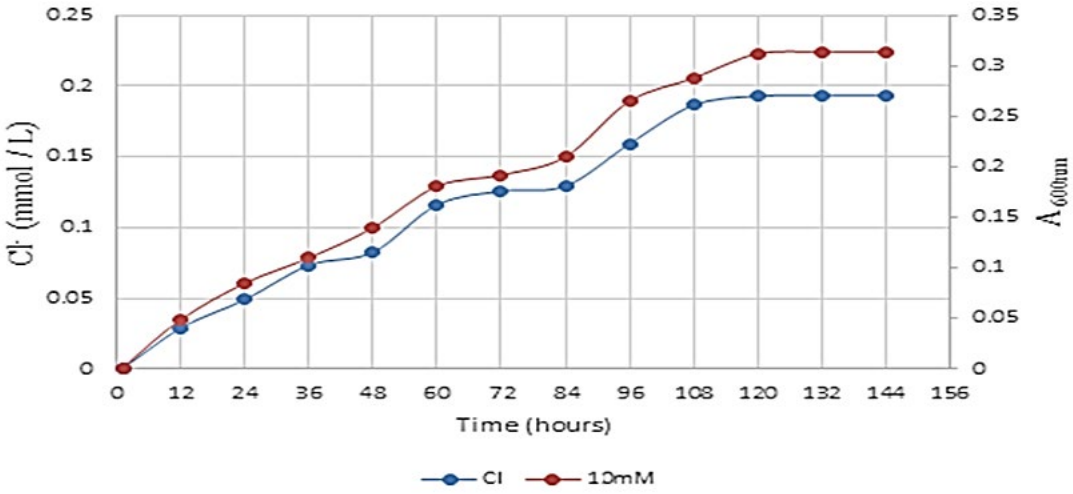


Figure 3. Correlation of 20 mM 2,2-DCP of WM growth with chloride ion released.

of 2,2-DCP. Bacterial growth on these media showed that the 2,2-DCP carbon atoms are suitable for use.

Growth profile: The growth profile of isolated bacteria strain WM in different substrate concentrations are presented in Figure 2.

Assay of halide ion: Ion liberation was controlled by halide ion to confirm the ability of WM bacteria to degrade 2,2-DCP. The chloride ion concentration was determined using mmol/L as standard measurements of soluble chloride by converting the absorption value into the concentration of sodium chloride (Bergmann and Sanik, 1957) showing the results in Figure 3.

DNA extraction and PCR amplification of 16S rRNA gene: To extract genomic DAN, the DNA extraction and purification kits (Promega, Wizard® Genomic DNA Kits) were used and after nano-drop analysis, and the concentration of the isolated genetic material was approximately 300 ng/µl. The 16S rRNA gene has been amplified using universal primers of 16s-f and 16s-r. Using gel electrophoresis, the PCR product was visualised, showing a gene with almost 1500 bp. A total of 1500 bases were aligned with other retrieved sequences, indicating that WM is a member

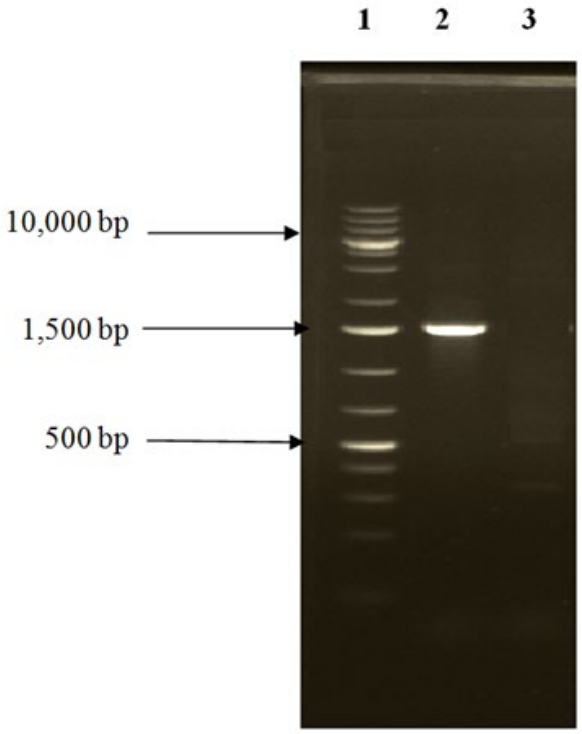


Figure 4. Gel electrophoresis of PCR product - 16S rRNA of bacterial strain WM. The PCR amplification of 16S rRNA gene on an agarose gel (1%), Lane 1: DNA Ladder (1 kb), Lane 2: Amplification of 16S rRNA gene from bacterium WM, Lane 3: Negative control (dH2O + 16S-f, 16S-r + PCR mix).

of the genus Enterobacter and its partial 16S rRNA gene sequence was submitted to NCBI GenBank (Fig. 4).

The 16S rRNA, is a universal target of highest precision for identification of bacteria (Ventura and Zink, 2002). Based on the results, bacterium WM is identical to *Enterobacter* sp., which corresponds to a sequence identity of 98%. The reconstructed phylogeny tree of the extracted 16S rRNA and those retrieved from NCBI with a bootstrap value based on NJ algorithm are shown in Figure 5.

As finding of this study, a soil-isolated bacterium has a great potential in degrading 2,2-DCP, identified as *Enterobacter* sp. that was well-growing in the minimum medium of 10, 20 and 30 mM. It produces the dehalogenase enzyme and as an environmentally friendly bacterial strain, can be useful as a bioremediation agent of detoxifying xenobiotic compounds through 2,2-DCP degradation.

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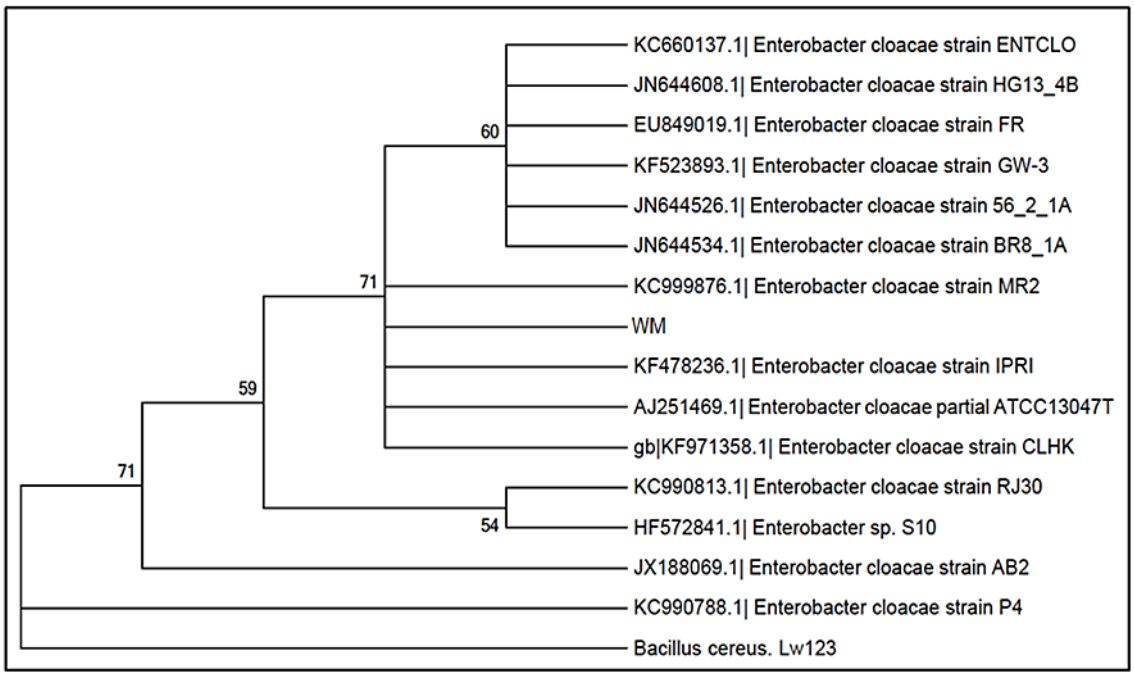

Figure 5. Neighbor-Joining phylogenic tree of WM isolate. The numbers at nodes are the bootstrap values.

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