ISSN: 2277-9655
Scientific Journal Impact Factor: 3.449
(ISRA), Impact Factor: 2.114

IJESRT

INTERNATIONAL JOURNAL OF ENGINEERING SCIENCES & RESEARCH TECHNOLOGY

# Isolation, Identification and Characterization of Thermo-tolerant Bacteria from Hot-Spring that can degrade Halogenated Compounds

**Yaseen Khashman Hussein* and Fahrul Hyup**

* Industrial Biotechnology Department, Faculty of Biosciences & Bioengineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia

*Ph.D Student

y.khashman@yahoo.com

## Abstracts

To investigate the microbial community in the hot spring of Krabi, Thailand and if they can degrade some halogenated compounds. A thermophilic bacterium (YKH) was isolated from the Krabi hot spring in Thailand. The isolate had entire and slimy colonies while the cells were small rods and gram-positive. The growth profile of YKH was measured at 60 $^{O}$C in different concentrations of 2,2 DCP (5 mM, 10 mM, 15 mM, 20 mM and 25 mM). The full length sequence with around 1220 base pairs of the sample YKH was obtained. Full sequence of YKH was blasted via National Center of Biotechnology Information (NCBI) to be compared with other known bacteria species and the similarities with other species were obtained. BLAST search result of partial 16S rRNA sequence obtained showed that YKH has close matches with *Bacillus* sp. with 100% and 99% similarities respectively. Result from BLAST suggested that strain YKH belongs to the genus *Bacillus* sp..The identification and the characterization are depending on the 16S rRNA and the morphological, biochemical tests respectively. The results of the BLAST search results represented by MEGA4 (phylogenetic tree). According to previous researches up to date, the study of degradation of halogenated compounds by thermophlic bacteria is very limited. This study focuses on the degradation of these compounds by thermo-tolerant bacteria. So, the results of this study allow us to make a library of the microorganisms which are able to degrade 2, 2-dichloropropionic and classify them based on genus and species analysis. These bacteria strains might be important for future study on the enzyme dehalogenase that can act at high temperature.

**Keywords:** Hot-spring- Thermophilic- Halogenated Compounds- *Bacillus* sp.-16S rRNA.

## Introduction

Many types of thermophilic and thermo-tolerant microorganisms are inhabitants of hot springs. According to **Wood Sworth, 1997,** the formation of hot springs come about when water penetrates through permeable rocks or fractures, heated by the earth's crusts at depth, and is then driven to the earth's surface by a combination of artesian flow and thermal convection. Hot waters are contain high levels of metals for instance, arsenic concentrations in water from hot springs in yellow stone national park have been documented to reach 1-6 mg/L able to soften minerals over time as reported by **Stauffer and others in1980.** Microbes that flourish in earth's severe environments are understood to be the probable candidates for the types of biota that may survive in extra terrestrial habitats.

A lot of extremophilic regions ranging from permafrost, hydrothermal vent, to evaporating crystals, are mainly located in Mars planet. As reported by **Edwards, 1990,** organisms growing at high temperatures, the thermophiles are in universally classified into three groups based on their temperature range of surviving (i) hyperthermophiles (75-113 $^{O}$C), (ii) extremophiles (55-85 $^{O}$C) and (iii) thermophiles (35-70 $^{O}$C). All ecosystems constitute of microorganisms and the survival of these organisms is due to their capability to grow and reproduce under aerobic or anaerobic conditions, small size and easily accelerated dispersal. Other cases are due to their metabolic multiplicity, tolerance to various environments and the ability to take possession under hostile spots case in point, those with extremes, redox, potentials, salinity, temperatures, humidity and pH. They are thus called extremophiles **(Stolp, 1988; Delong 1988).**

According to **Hard Wood, 1989,** thermophilic bacteria are source of thermostable enzymes which has shaped much interest in learning them by the present scientists. The resistance to denaturation in organic solvents is referred to as the thermostability. Thermostable enzymes are expected to encompass properties suitable for many industrial applications.

ISSN: 2277-9655
Scientific Journal Impact Factor: 3.449
(ISRA), Impact Factor: 2.114

Enzymes from extremophiles propose some important possible profits ranging from spoilage removal of sulfur from coal and oil, sugar conversions and the modification of flavor or texture of foods without microbial growth.

A wide range of bacterial species that can degrade such substances and, in many cases, utilizes them as sole sources of carbon and energy have been isolated in laboratory culture, after selection and genetic manipulation, such strains can be used to facilitate decontamination. Therefore, we need to understand much more about the process of microbial adaptation involved in order to harness this potential. Some of these thermophilic bacteria can degrade theses halogenated compounds by producing the specific dehalogenase enzyme and utilize them as a carbon and energy source. This study focused on the isolation, identification and characterization of selected bacteria from hot spring sample. The ability of this bacteria to degrade herbicide pollutant such as 2,2 DCP was investigated **(Owusu and Cowan, 1989).**
The aim of this study to isolate and identify thermophilic bacteria in krabi, Thailand to find a thermo-tolerant bacteria that can degrade specific halogenated compounds (2,2 DCP) at high temperature degree.

## Materials and methods

**Sample collection:** Soil sample was taken from the hot spring, Krabi Thailand by Petri dishes. The sample was randomly collected from the hot spring area.

**Isolation of thermophilic bacteria:** Thermophilic bacteria were isolated on Petri plates containing minimal agar medium (The formulation of agar minimal media as in Table: 1 bellow) through serial dilution of the samples. One hundred microlitre of the sample was poured over the media and incubated at 60 $^{O}$C while same quantity of sample was inoculated to the flask containing LB medium to isolate thermophiles **(Narayan *et al*., 2008).**

***Table-1: The formulation of agar minimal media.***

| Compound | Flask A | Flask B |
|---|---|---|
| Distilled water(mL) | 30 | 46 |
| Agar powder (g) | - | 3 |
| Basal salts solution (10x) (mL) | 10 | - |
| Trace metal salts solution (10x) (mL) | 10 | - |

**Morphological and biochemical characterization:**
The morphology and biochemical tests were carried out according to **MacFaddin (2000)** to determine the characteristic of the pure couture. The morphology tests include Gram staining and biochemical tests which involved various methods such as catalase test, lactose utilization test, oxidase test and urease test.

**Kynticks study**
The growth curve was performed to determine the growth of microorganisms in the defined medium that contains only one carbon source. The growth was measured using spectrophotometer at $A_{600nm}$ and in different concentrations of 2,2 DCP (5 mM, 10 mM, 15 mM, 20 mM and 25 mM).

**Molecular characterization**
Genomic DNA was extracted from the bacterial cells (overnight culture). Wizard genomic purification Kit (Promega) was used according to manufactures instruction. The 16S rRNA was amplified from purified DNA by PCR using pfu DNA polymerase with the buffer supplied by the manufacture promega. Universal 16S rDNA were synthesized by 1$^{st}$ BASE Laboratory Malaysia Sdn. Bhd. The procedures was carried out according to **Ng and Huyop (2007) and Cappuccino and Sherman (2002).** The forward primer, Fd1 sequence:
"5'-AGA GTT TGA TCC TGG CTC AG-3' "
While the nucleotide sequence for the reverse primer rp1:
"5'-ACG GCT ACC TTG TTA CGA CTT-3' ". PCR master mix from "1st base company" was used in PCR reaction. PCR amplification was carried out with 1 µl

ISSN: 2277-9655
Scientific Journal Impact Factor: 3.449
(ISRA), Impact Factor: 2.114

(50ng) DNA as a template, 5 µl of 2.5 mM dNTPs, l µl of 10 µM of each primer, 5 µl of 25 mM magnesium chloride, 5 µl of IX PCR buffer (75 mM Tris-HCl pH 8.8 at 25°C, 20 mM (NH4)2SO4 and 0.01% tween 20) and l µl of 2.5 units of Taq DNA polymerase in a 50 µl reaction mixture. The reaction mixture was incubated at 94°C for 5 min and then subjected to 35 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 1 min; followed by one final cycle of 72°C for 10 min. The PCR product was analysed by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. The PCR product was purified using spin prep gelmelt kit (Novagen). The purified 16S rRNA was then sequenced using 3130xl Genetic Analyzer. Highest similarities of the 16S rRNA gene sequence of YKH to that of other bacteria were searched using the BLAST tool of GenBank **(Altschul *et al.*, 1990).** The 16S rRNA sequence of strain YKH was aligned with representative 16S rRNA sequences of related taxa using Clustal W software **(Thompson *et al.*, 1994).** Phylogenetic tree was constructed using neighborjoining method **(Saitou and Nei, 1987)** and Jukes and Cantor model **(Jukes and Cantor, 1969)** employing the software MEGA 4.0 **(Tamura *et al.*, 2007).** The stability of relationships was assessed by performing bootstrap analysis of the neighbor-joining data based on 1000 resamplings.

**Nucleotide sequence accession number**

The 16S rRNA sequence of strain YKH has been deposited in the GenBank database under accession no. JF900046.1.

## Results and discussion

**Isolation of bacteria**

Bacteria were isolated from the soil sample taken from Krabi- hot spring (Thailand). Isolate was cultivated on minimal media containing 10 mM 2, 2 dichloropropionate as sole source of carbon and energy (without yeast extract added). Bacteria colony emerged after 72 hours along with incubation at 60 $^{O}$C. The isolate formed circular, white and creamy colonies on agar minimal media. (Fig: 1).

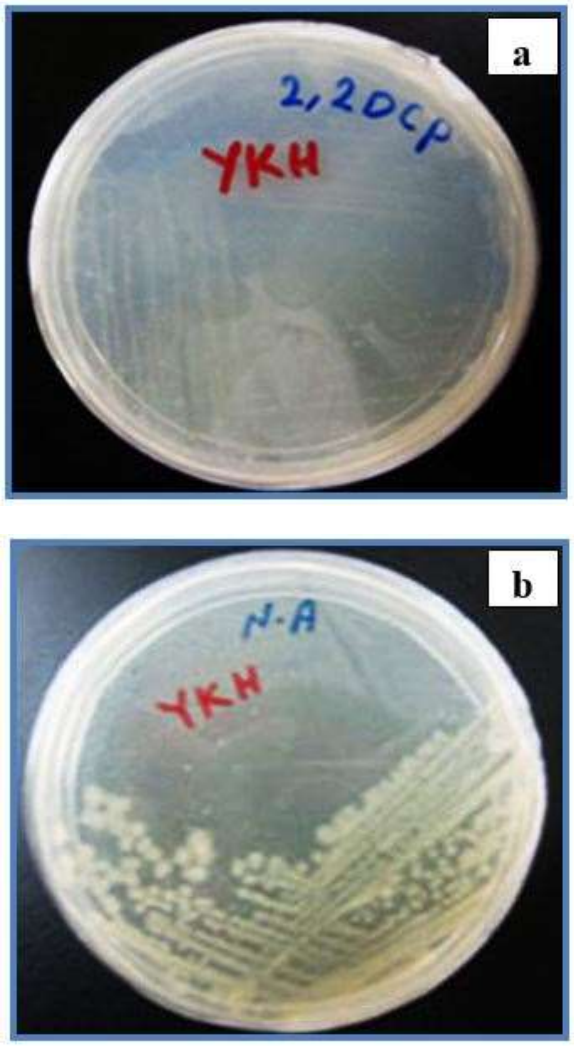


***Fig-1: (a). Shows colony morphology in 10mM 2,2 DCP plate grown after 72 hours at 60 $^{O}$C. (b). Pure colonies morphology of YKH in agar media.***

**Identification and characterization of thermophilic bacteria (YKH)**

***Table-2: summarizes all the observations. Control plate using E. coli was used and was grown in similar conditions with the isolated organism. However, no growth was observed.***

| Characteristics | Observation |
|---|---|
| Strain | YKH |
| Time incubation | 72 hours |
| Size (mm) | Small (0.5 ~1 mm) |
| Colour | Creamy to white |
| Edge | Raised |
| Shape | Round |
| Motility test | Positive/turbidity diffused away from the stab line |

ISSN: 2277-9655
Scientific Journal Impact Factor: 3.449
(ISRA), Impact Factor: 2.114

The morphology and biochemical tests were carried out to determine the characteristic of the pure couture. The morphology tests include Gram staining and different biochemical tests. To identify and characterize the isolated microorganism, a few of biochemical tests had been performed. They were: Catalase test, Citrate test, Lactose Utilizatio test, Oxidase test and Urease test as summarized in (Table-3 and Fig: 2).

***Table-3: The result that obtained from Biochemical analysis and gram staining***

| Biochemical test | Result | Observation |
|---|---|---|
| Catalase Test | Positive | Gas bubbles were released |
| Citrate test | Negative | No change colour |
| Lactose Utilization Test | Negative | No Growth observed |
| Oxidase Test | Positive | Change to violate colour |
| Urease Test | Positive | Medium changed colour from yellow to pink |
| Gram staining | Gram Positive | Rod in shape with blue color |

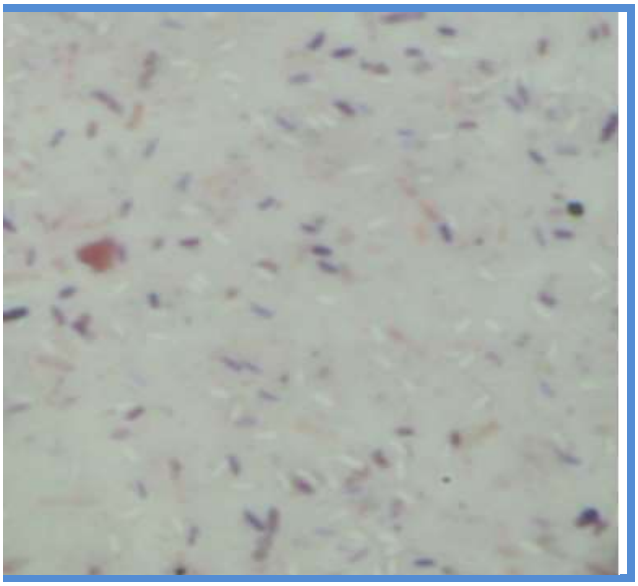

***Fig-2:Microscopic observation of YKH.***

**Growth Profile (kinetics study)**

**Growth Profile of YKH in nutrient broth**

To test the biodegradation of 2, 2 DCP in different concentration, initially the bacterium YKH was grown in nutrient broth for 30 hours at 60°C. Thus, to determine the maximum growth (log phase). The measurement of optical density at $A_{600nm}$ was taken every two hours. The results showed that the maximum growth was detected between 12-16 hours. The highest of the maximum growth was observed at 12 hours (1.947 $h^{-1}$). The experiment was repeated to take the bacterium after 12 hours to inoculate it in a minimal media containing different concentration of 2,2 DCP as shown in (Fig: 3).

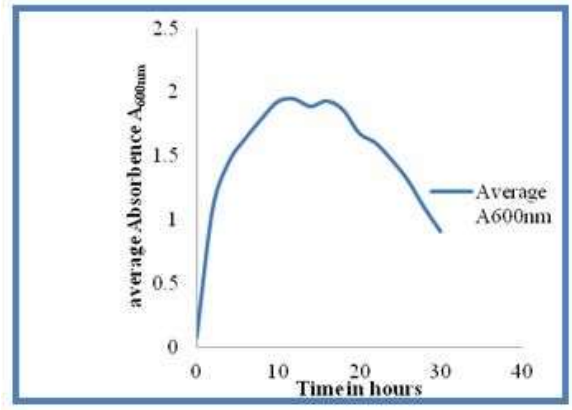


***Fig-3: YKH growth in nutrient broth media***

**Growth Profile of YKH in minimal media**

The growth profile was constructed by determine the bacterial growth rate and different growth stages. Bacteria YKH was grew in duplicate minimal liquid media using temperature-adjustable shaker at 60 $^{O}$C, 200 rpm. Five different concentration, 5mM, 10mM, 15mM, 20mM and 25mM of 2, 2-dichloropropionate as sole carbon source has been used to determine the growth rate of bacterium in these different condition. From the first attempt, the bacterium was found to grow very slowly as started with inoculation using bacterial colony into liquid minimal media, which suggesting the carbon source was toxic to the bacteria **(Anwar *et al*., 2009).** Measurement of optical density at $A_{600nm}$ was taken at 6 hours interval. (Fig: 4) below showed reading at $A_{600nm}$ taken every 6 hours until 60hours.

ISSN: 2277-9655
Scientific Journal Impact Factor: 3.449
(ISRA), Impact Factor: 2.114

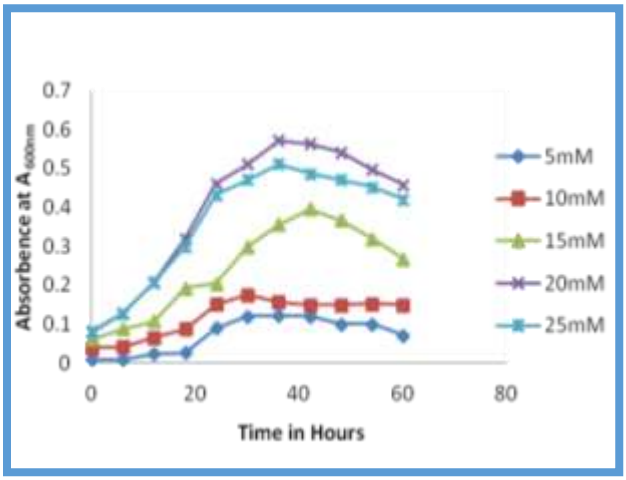


***Fig-4: Showing average $A_{600nm}$ for YKH grown in the different concentrations of 2,2-DCP (60 $^{O}$C).***

The maximum growth was attained in 20 mM concentration ($A_{600nm}$ =0.573) while least growth was observed in 5 mM concentration ($A_{600nM}$ = 0.122). 15 mM and 25 mM concentrations could also support the growth of YKH but the maximum growth obtained in both cases ($A_{600nm}$ = 0.396 and $A_{600nm}$= 0.512) were less than that of 20 mM. Bacteria did not grow well in 5 mM 2, 2- DCP suggesting the growth was inhibited due to less substrate availability. The higher the concentration of the substrate the higher the nutrient capacity it holds and vice versa (**Zeng, and Deckwer, 1995).**

Although absorbance was low at $A_{600}$nm, visually the difference in turbidity cause by bacterial grow among different concentration of 2,2-dichloropropionate was noticeable, with highest cloudiness in media with 20 mM 2,2-DCP, followed by 25 mM 2,2-DCP, 15 mM 2,2-DCP , 10 mM 2,2-DCP and it was least observed in 5 mM 2,2-DCP showing least light penetration while media with 5 mM 2,2-DCP. The lower maximum growth in 25 mM than in 20mM 2,2-DCP could be due to increased level of the toxicity **(Cabrera *et al.*, 2006).** Bacteria have the optimum concentration over which they can work best. So in this case, 20 mM 2,2-DCP proves to be optimum concentration for YKH growth as the maximum growth was observed at $A_{600}$nm= 0.573).
Doubling time of bacteria in different concentration of 2,2-dichloropropionic acid was also determined using the formula In/μ where μ is the growth rate. The results are summarized as shown in (Table-4).

***Table-4: Showing the growth rate of YKH in different concentrations***

| Concentration | Growth rate ($h^{-1}$) | Doubling time (In2/growth rate) |
|---|---|---|
| 5mM | 0.084 | 8.25h |
| 10mM | 0.089 | 7.79h |
| 15mM | 0.033 | 21h |
| 20mM | 0.025 | 27.72h |
| 25mM | 0.028 | 24.75h |

From the table above, it can be seen that the lower the concentration of the substrate, the higher the growth rate, thus YKH had a highest growth rate ($A_{600nm}$= 0.089) when grown in 10 mM 2,2-DCP. This is because the lower the substrate concentration, the less the toxicity and thus the faster the degradation of substrate **(Ullrich *et al.*, 1996).** This provides easy accessibility of the bacterium to the substrate and thus faster growth rate.

Calculated average doubling time of bacteria in 20mM 2,2-DCP was 27.72 hours compared to 5mM, 10mM, 15mM and 25mM 2,2-DCP, which were 8.25h, 7.79h, 21h and 24.75h (Table-4). Since maximum absorbance in 5mM 2,2-DCP was too low and low obvious growth stage was recorded, therefore considered not good for the bacterial grown in this media. The effect of concentration on the growth profile of YKH can be clearly observed by plotting the growth rate against the different concentrations of 2,2-DCP.

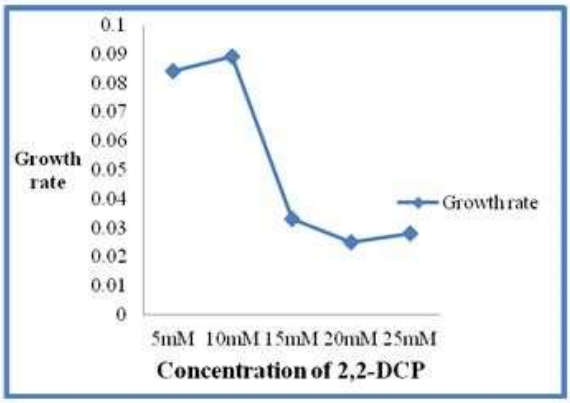


***Fig-5: showing the effect of concentrations of 2,2-DCP on the growth rate of YKH***

ISSN: 2277-9655
Scientific Journal Impact Factor: 3.449
(ISRA), Impact Factor: 2.114

Generally the growth rates were higher when low concentrations of 2, 2-DCP were used (5mM and 10mM). This is due to easy availability of the substrate to the bacterium in less concentrated substrate than in the highly concentrated substrate **(Kovárová-Kovar and Thomas, 1998)** due to increased toxicity **(Cabrera *et al.*, 2006)** and thus making it for the bacterium to take longer time of degradation. At 15 mM concentration, the growth rate drops, indicating that 15 mM is the Minimum Inhibitory Concentration (MIC) for the growth of YKH in 2,2 DCP.

**16S rRNA gene analysis results**

Bacteria YKH was subjected to this analysis. Details as described in the next sections.

**1-Genomic DNA Extraction Result**

Genomic extraction result was shown in (Fig: 6).This experiment suggested that the genomic DNA was successfully prepared.

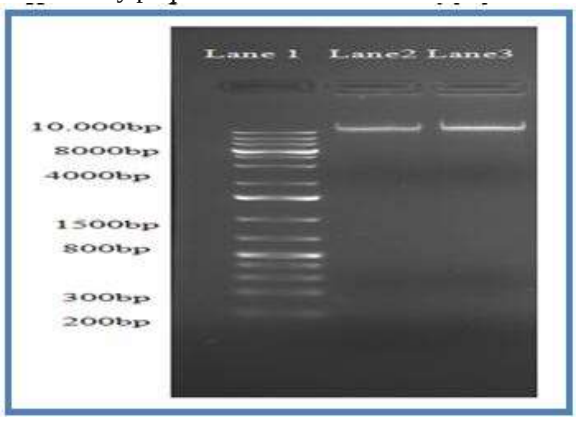


*Fig-6: DNA gel electrophoresis under UV light*
*Lane1: 1kb ruler*
***Lane2: The extracted Genomic DNA of (YKH). (1.9 ng/µL)***
***Lane3: The extracted Genomic DNA of (YKH). (1.9 ng/µL)***

**2-Polymerase Chain Reaction (PCR) Result**

In order to amplify the gene 16S rRNA, Polymerase Chain Reaction (PCR) was carried out. DNA genomic of bacterium YKH was used as templates. PCR products were tested using gel electrophoresis in order to assure that the amplification was carried out successfully. The amplified 16S rRNA gene with the length of 1500bp was observed in (Fig: 7).

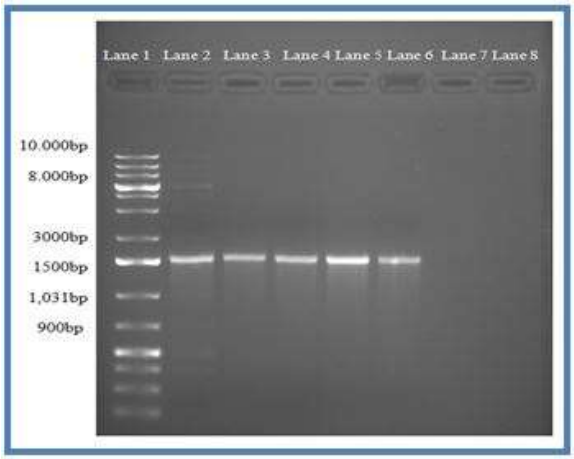


*Fig-7: PCR product for 16SrRNA (gel electrophoresis under UV light)*
***Lane1: 1kb ruler***
***Lane2-6: PCR product of 16S rRNA***
***Lane7: Control: without Fd1 primer***
***Lane8: Control: without rp1 primer***

**3 -PCR purification**

PCR product was purified by using QIAquick Nucleotide Removal Kit. This was to ensure the removal of primers, enzymes, salts, and unincorporated nucleotides. After purification, the gel electrophoresis was carried out to assure the availability of the purified PCR products. (Fig: 8).

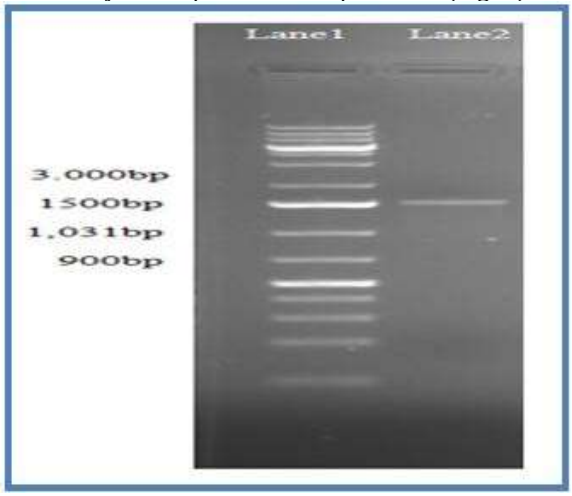


*Fig-8: Gel electrophoresis of purified PCR product.*
*Lane1: 1kb ruler*
***Lane2: Purified PCR product of YKH***

DNA band at 1500bp indicated the product of PCR was ready for sequencing. The clear single band in the gel electrophoresis shows the sufficient concentration of PCR product. Based on the figure above, sample YKH showed a lighten band at the

ISSN: 2277-9655
Scientific Journal Impact Factor: 3.449
(ISRA), Impact Factor: 2.114

size of 1500pb. PCR product was launched for DNA sequencing.

**4-16S rRNA sequence (computational analysis)**

The disinfected PCR product was sent to the 1st Base ® Company for DNA sequencing, the outcome of sequencing had been received through e-mail in ".ab1" format. The YKH files were viewed using Sequence scanner software.

Result from BLAST was shown as in (Fig: 9). The identity suggests strain YKH belongs to the genus *Bacillus* sp.

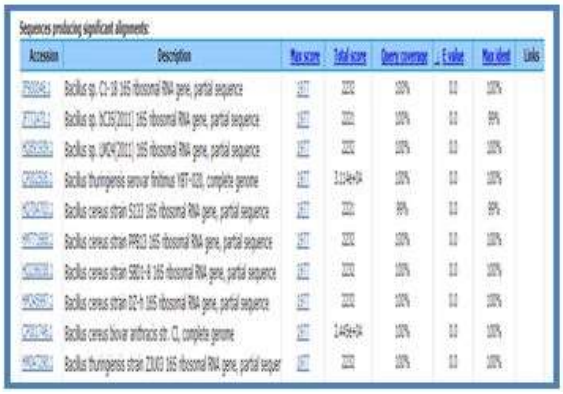

***Fig-9: Top ten BLAST search result of YKH.***

As overall, BLAST search result of partial 16S rRNA sequence obtained showed that YKH has close matches with *Bacillus* sp. with 100% and 99% similarities with *Bacillus* sp, C1-18 165 and bc35 respectively.

**5-Phylogenetic among dehalogenase producing bacteria**

The alignment was performed using MEGA4 version 4.0.2 and the topology of the tree was constructed by neighbor- joining method. Ten candidates were chosen from other dehalogenase producing bacteria that can degrade halogenated compounds. A rooted phylogenetic tree which shows the relationship between the ten bacteria and YKH was represented in (Fig: 10)

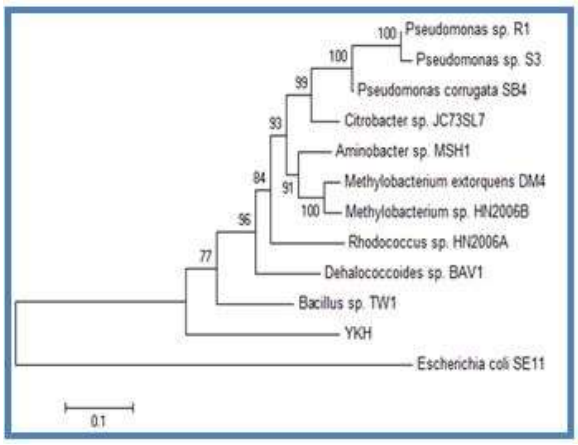


***Fig-10: Rooted phylogenetic tree (triangular) shows the relationship between identified bacteria YKH and other dehalogenase producing bacteria that can degrade halogenated compounds.***

The results showed strain YKH has close relationship with *Bacillus* sp. TW1 as expected TW1 has the ability to utilize the halogenated compounds as a carbon and energy source, which may possibly confirm the ability of YKH to degrade 2,2 DCP **(Zulkifly *et al*., 2010).**

## Conclusion

In this study, *Bacillus* sp. strain YKH was isolated from the soil sample taken from hot spring in Krabi, Thailand. According to previous researches up to date, the study of degradation of halogenated compounds by thermophlic bacteria is very limited. In this study, the isolated showed a high ability to degrade halogenated compounds as a carbon and energy source. So, this study suggested that this theromphilic bacterium has the capability to degrade these halogenated compounds. Beside, the results of this work can help to make a library for microorganisms that can produce dehalogenase enzyme which can act at high temperature. So, this enzyme can be used in many industrial and medical applications.

## Acknowledgment

The author thanks to the Malaysian Government and Universiti Teknologi Malaysia for the help in this work. Appreciation and thanks to all staff of Faculty of Biosciences and Bioengineering– Microbiology and molecular Biology lab- UTM, Malaysia for encouragements and support during this research work.

## References

1. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990).** Basic local alignment search tool. J Mol Biol.;215:403–410.

ISSN: 2277-9655
Scientific Journal Impact Factor: 3.449
(ISRA), Impact Factor: 2.114

2. **Anwar, S., Liaquat, F., Khan, Q. M., Khalid, Z. M., & Iqbal, S. (2009).** Biodegradation of chlorpyrifos and its hydrolysis product 3, 5, 6-trichloro-2-pyridinol by< i> Bacillus pumilus</i> strain C2A1. *Journal of Hazardous Materials*, *168*(1), 400-405.
3. **Cabrera, G., Pérez, R., Gomez, J. M., Abalos, A., & Cantero, D. (2006).** Toxic effects of dissolved heavy metals on< i> Desulfovibrio vulgaris</i> and< i> Desulfovibrio</i> sp. strains. *Journal of hazardous materials*, *135*(1), 40-46.
4. **Cappuccino, J. G. and Sherman, N. (2002).** Microbiology: A Laboratory Manual (6th edition). Pearson Education, Inc. San Francisco, California.
5. **Delong, E. (1998).** Archaeal means and extremes. Science 280, 542-543. DOI: 10.1126/science.280.5363.542.
6. **Edwards, C. (1990).** Microbiology of Extreme Environments. McGraw-Hill, New York.
7. **Hardwood, C. R.(1989).** Bcillus, vol. 2 . Biotechnology Hndbooks. Plenum Press, London. lacumin, L., Coml, C., Cocolin.
8. **http://www.megasoftware.net**/WebHelp/part_iv__evolutionary_analysis/c
9. **Kovárová-Kovar, K., and Egli, T. (1998).** Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. *Microbiology and molecular biology reviews*, *62*(3), 646-666.
10. **MacFaddin, J. F. (2000).** Biochemical tests for identification of medical bacteria, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA..
11. **Narayan VV, Hatha MA, Morgan HW, Rao D. (2008).** Isolation and characterization of aerobic thermophilic bacteria from Savusavu hot springs in Fiji. Microbes Environ.23:350–352.
12. **Ng, H. J. and Huyop, F. Z. (2007).** Dehalogenation Of Chlorinated Aliphatic Acid By Rhodococcus Sp., Asia Pacific Journal of Molecular Biology and Biotechnology, Volume. 15 (3). p147-151.
13. **Computing_evolutionary**_distances/distance_models/nucleotide_substitution_models/hc_jukes_cantor_distance.htm.
14. **Owusu, R.K., Cowan, D.A.** (**1989).** A correlation between microbial protein thermostability and resistance to denaturation in aqueous-organic solvent two-phase systems. Enzyme and Microbial Technology. 11, n568-574. p. 227-236. DOI: 10.1016/0141-0229(89)90084-7.
15. **Saitou, N., and Nei, M. (1987).** The Neighbor-Joining Method: A New Method for Reconstructing Phylogenetic Trees. Molecular Biology and Evolution 4:406 425.
16. **Stauffer RE, Jenne EA, Ball JW. (1980).** Chemical studies of selected trace elements in hot-spring drainages of Yellowstone National Park. Geological survey paper 1044-F. Washington: United States Government.
17. **Stolp, H.(1988).** Microbial Ecology: Organisms, Habitats, Activities. Cambridge University Press, Cambridge. ISBN-10: 0521256577 | ISBN-13: 978-0521256575.
18. **Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007).** MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Molecular Biology and Evolution 2007 24(8):1596-1599.
19. **Thompson JD[1], Higgins DG, Gibson TJ. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 11;22(22):4673-80.
20. **Ullrich, S., Karrasch, B., Hoppe, H., Jeskulke, K., & Mehrens, M. (1996).** Toxic effects on bacterial metabolism of the redox dye 5-cyano-2, 3-ditolyl tetrazolium chloride. *Applied and environmental microbiology*, *62*(12), 4587-4593.
21. **Woodsworth G. (1997).** Hotsprings of western Canada. West Vancouver, Canada: Gordon Soules. 14-15.
22. **Zeng, A. P., and Deckwer, W. D. (1995).** A kinetic model for substrate and energy consumption of microbial growth under substrate-sufficient conditions. *Biotechnology progress*, *11*(1), 71-79.
23. **Zulkifly,A.H., D.D.Roslan, A.A.A.Hamid, S.Hamdan and F.Huyop. (2010).**Biodegradation of low Concetration of Monochloroacetic Acid degrading Bacillus sp. TW1 Isolated from Terengganu Water Treatment and Distribution Plant. Journal of Applied Sciences.,10(22):2940-2944. DOI: 10.3923/jas..2940.2944.