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Identification of Cellulose-Hydrolytic Thermophiles Isolated from Sg. Klah Hot Spring Based On 16S rDNA Gene Sequence

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***Abstract*—**In this study, six bacterial isolates of a slightly thermophilic organism from the Sg. Klah hot spring, Malaysia were successfully isolated and designated as M7T55D1, M7T55D2, M7T55D3, M7T53D1, M7T53D2 and M7T53D3 respectively. The bacterial isolates were screened for their cellulose hydrolytic ability on Carboxymethlycellulose agar medium. The isolated bacterial strains were identified morphologically, biochemically and molecularly with the aid of 16S rDNA sequencing. All of the bacteria showed their optimum growth at a slightly alkaline pH of 7.5 with a temperature of 55°C. All strains were Gram-negative, non-spore forming type, strictly aerobic, catalase-positive and oxidase-positive with the ability to produce thermostable cellulase. Based on BLASTn results, bacterial isolates of M7T55D2 and M7T53D1 gave the highest homology (97%) with similarity to *Tepidimonas ignava* while isolates M7T55D1, M7T55D3, M7T53D2 and M7T53D3 showed their closest homology (97%-98%) with *Tepidimonas thermarum*. These cellulolytic thermophiles might have a commercial potential to produce valuable thermostable cellulase.

***Keywords*—**Cellulase, Cellulolytic, Thermophiles, 16S rDNA Gene.

I. Introduction

THERMOPHILES can be defined as microorganisms that can withstand, survive and capable of catalyzing biochemical reactions at high temperatures [1]. This is due to the fact that they have specialized proteins called chaperonins that can resist denaturation and proteolysis. Thermostable enzymes are highly specific and thus have considerable potential for many industrial applications. The use of such enzymes in maximizing reactions accomplished in the food and paper industry, detergents, drugs, toxic wastes removal and drilling for oil is being studied extensively. Through either optimized fermentation of the microorganisms or cloning of fast-growing mesophiles by recombinant DNA technology, the enzymes can be produced by the thermophiles [2]. Thermozymes generally posses several advantages as they are more resistant towards harsh conditions such as extreme low or high pH, detergents, organic solvents and other denaturing agent compared to their mesophilic counterparts [3].

Cellulase is the enzyme that hydrolyzes the β-1,4-glycosidic bonds in the polymer to release glucose units [4]. At present, thermostable cellulases are used in various food, brewery and wine industry, textile and laundry, pulp and paper industries, as well as in agriculture and for research purposes [5], [6]. Application of cellulase enzyme includes the polymer degradation in detergents and for cellulose hydrolysis [7].

Bioprospecting is defined as the exploration of biodiversity for commercially valuable biochemical and genetic resources for achieving economic and conservation goals [8]. In any industrial applications, enzymes that are cheap and readily available in biomass, renewable, can operate in high temperature, and better substrate solubility is highly demanded. It has been reported that thermophiles are the sources of industrially relevant thermostable enzymes and there is an increasing need of it in industrial applications [9]. Hence, the isolation and identification of novel celluloytic-hydrolytic thermophiles from natural sources are beneficial in terms of discovering potential industrially important thermo tolerant cellulases.

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II. Materials and Methods

A. Study Site & Collection of Samples

Water samples were collected from Sg Klah Hot Spring Park located in Perak, Malaysia (03º59'40N and 101º23'33E). The temperature of the hot spring water within collection time is 91.0-92.5ºC. The pH value of the thermal springs is 8.68-8.71 (alkaline) with percentage of dissolved oxygen at 28.1%. Water samples were kept in thermos to maintain the temperature and transported to the lab within 5 hours.

B. Qualitative Screening of Cellulolytic Activity

Thermophilic bacteria were screened for cellulolytic activity on Carboxymethylcellulose agar (CMC) agar containing: 5.0 gram of sodium chloride, 0.1 gram of calcium chloride dehydrate ($CaCl_2.2H_2O$), 10.0 gram of peptone, 20.0 gram of agar and 2.0 gram of CMC. The pH of medium medium was adjusted to pH 7.0 using 1M of sodium hydroxide (NaOH). The streaked plates were then incubated at 55°C for 1-2 days. The plates were flooded with Gram's iodine and halo zone formation indicated positive cellulose hydrolysis activity [10].

C. Morphological & Biochemical Test

Morphological characteristics of isolates were identified using Gram staining techniques and colony morphology. The cultures were maintained on nutrient agar slants at 4°C. Positive isolates were also evaluated by few biochemical tests: catalase, oxidase and starch hydrolysis and carbohydrate

fermentation.

D. Genomic Extraction and 16S rDNA Gene Amplification

DNA was extracted from bacteria with DNeasy Blood and Tissue Kit (QIAGEN, USA). Amplification of the 16S rDNA genes was performed with a pair of universal primer of 63F (5'- CAGGCCTAACACATGCAAGTC – 3') and 1389R (5' – ACGGGCGGTGTGTACAAG – 3') [11]. The following thermal profile was used: denaturation at 94°C for 1 min, primer annealing at 47.5°C for 30 sec and extension at 72°C for 30 sec. The final cycle included extension for 15 min at 72°C to ensure full extension of the products.

E. 16S rDNA Gene Analysis

Amplified PCR products were sequenced at 1st BASE Laboratory Sdn. Bhd. (Selangor, MY). Partial sequences obtained were aligned and compared with sequence from GenBank using BLASTn. The unknown bacterium was also further identified using bioinformatics' tools; SEQ MATCH and RDP Classifier from the Ribosomal Database Project (RDP).

III. Results and Discussion

A. Isolation and Screening of Cellulolytic Thermophiles

A total of six thermophillic bacteria with the capability of utilizing carboxymethylcellulase (CMC) as their sole carbon source were successfully isolated and denoted as M7T55D1, M7T55D2, M7T55D3, M7T53D1, M7T53D2 and M7T53D3 respectively. These isolates produced variable zones of CMC clearance.

B. Colony Morphology of the Cellulolytic Isolates

All of the colonies were observed to be smooth surface with undulated edge and a bit of opaque in color. However, the bacterial isolates of M7T55D2 and M7T53D1 were found to be in irregular forms, and flat elevation with white abundant growth. Whereas, the bacterial isolates of M7T55D1, M7T55D3, M7T53D1 and M7T53D2 were observed to be in circular form and convex elevation. It has been observed that isolates having almost identical 16S rDNA sequences displayed slight colony morphological diversity, even under uniform growth conditions.

C. 16S rDNA Genes Alignment

The 16S rDNA sequences obtained from all the isolates were aligned with other closely related bacterial 16S rDNA sequences obtained from the GenBank by Basic Local Alignment Search Tool (BLAST) program accessed via the National Center for Biotechnology Information (NCBI) website. The 16S rDNA sequence alignment revealed that all of the isolates derived from the same genus of Tepidimonas as shown in Tables I and II. The results were corroborated by the result shown by the RDP Classifier where it clarifies that all of the isolates belong to domain Bacteria, phylum Proteobacteria, class Betaproteobacteria, order Burkholderiales, family Burkholderiaceae and genus Tepidimonas.

The species of the genus Tepidimonas, like the bacterial isolates of M7T55D1, M7T55D2, M7T55D3, M7T53D1, M7T53D2 and M7T53D3 have been primarily isolated from natural geothermally heated aquatic environments. The species of the genus Tepidimonas, namely *Tepidimonas thermarum*, *Tepidimonas ignava*, *Tepidimonas aquatica* and *Tepidimonas taiwanensis*, were isolated from public bath, hot springs and from an industrial hot water tank, respectively [12]-[15]. These Betaproteobacteria have optimum growth temperatures around 50 °C and two of these species do not utilize carbohydrates as single carbon sources. Based on 16S rDNA sequence, both bacterial isolates of M7T55D2 and M7T53D1 shared high percentage of homology with *Tepidimonas ignava* strain SPS-1037 with the accession number of NR 02504.1.1 at 97% identity (Table I). These two bacterial isolates also showed differences in cell and colony morphologies as compared to other bacterial isolates. In a study done by [14], *Tepidimonas ignava* sp. nov. is described as rod-shaped cells bacteria that stain Gram-negative. They do not form endospores and characterized as slightly thermophilic, strictly aerobic and positive for cytochrome oxidase and catalase. By comparison, these descriptions are similar to the characteristics of the bacterial isolates of M7T55D2 and M7T53D1 that has been successfully isolated from Sg. Klah hot spring.

TABLE I
BLASTn Analysis of **M7T55D2 and M7T53D1**

Thermophilic Isolates[a]	***% identity*** [a]	***Primer***	***Closest Phylogenetic Relative GenBank Accession No.***
M7T55D2	97	63F	*Tepidimonas ignava* strain SPS-1037 (NR 025041.1)
	97		*Tepidimonas thermarum* strain AA1 (NR 042418.1)
	96		*Tepidimonas taiwanensis* strain |1-1 (NR 042418.1)
	97	1389R	*Tepidimonas ignava* strain SPS-1037 (NR 025041.1)
	96		*Tepidimonas thermarum* strain AA1 (NR 042418.1)
	96		*Tepidimonas taiwanensis* strain |1-1 (NR 042418.1)
M7T53D1	97	63F	*Tepidimonas ignava* strain SPS-1037 (NR 025041.1
	97		*Tepidimonas thermarum* strain AA1 (NR 042418.1)
	96		*Tepidimonas taiwanensis strain |1-1 (NR 042418.1)*
	95	1389R	*Tepidimonas ignava* strain SPS-1037 (NR 025041.1
	95		*Tepidimonas thermarum* strain AA1 (NR 042418.1)
	90		*Tepidimonas taiwanensis strain |1-1 (NR 042418.1)*

[a]The percentage identity refers to percentage of similarity to 16S rDNA sequence.

On another note, data analysis of all the 16S sequences derived from M7T55D1, M7T55D3, M7T53D2 and M7T53D3 showed that all of these bacterial isolates shares closest homology with *Tepidimonas thermarum* strain AA1 (NR 042418.1) with the percentage of identity that fell within a range of 97 to 98% (Table II).

TABLE II
BLASTn ANALYSIS OF **M7T55D1**, **M7T55D3**, **M7T53D2** AND **M7T53D3**

Thermophilic Isolates[a]	*% identity*[a]	*Primer*	*Closest Phylogenetic Relative GenBank Accession No.*
M7T55D1	97	63F	*Tepidimonas thermarum* strain AA1 (NR 042418.1)
	97		*Tepidimonas ignava* strain SPS-1037 (NR 025041.1)
	97		*Tedimonas aquatic* strain CLN-1 (NR 025755.1)
	97	1389R	*Tepidimonas thermarum* strain AA1 (NR 042418.1)
	97		*Tepidimonas ignava* strain SPS-1037 (NR 025041.1)
	97		*Tedimonas aquatic* strain CLN-1 (NR 025755.1)
M7T55D3	98	63F	*Tepidimonas thermarum* strain AA1 (NR 042418.1)
	97		*Tepidimonas ignava* strain SPS-1037 (NR 025041.1)
	97		*Tepidimonas taiwanensis strain |1-1 (NR 042418.1)*
	94	1389R	*Tepidimonas thermarum* strain AA1 (NR 042418.1)
	94		*Tepidimonas ignava* strain SPS-1037 (NR 025041.1)
	94		*Tepidimonas taiwanensis strain |1-1 (NR 042418.1)*
M7T53D2	97	63F	*Tepidimonas thermarum* strain AA1 (NR 042418.1)
	95		*Tepidimonas ignava* strain SPS-1037 (NR 025041.1)
	95		*Tepidimonas taiwanensis strain |1-1 (NR 042418.1)*
	95	1389R	*Tepidimonas thermarum* strain AA1 (NR 042418.1)
	95		*Tepidimonas ignava* strain SPS-1037 (NR 025041.1)
	94		*Tepidimonas taiwanensis strain |1-1 (NR 042418.1)*
M7T53D3	98	63F	*Tepidimonas thermarum* strain AA1 (NR 042418.1)
	97		*Tepidimonas ignava* strain SPS-1037 (NR 025041.1)
	97		*Tepidimonas taiwanensis strain |1-1 (NR 042418.1)*
	98	1389R	*Tepidimonas thermarum* strain AA1 (NR 042418.1)
	97		*Tepidimonas ignava* strain SPS-1037 (NR 025041.1)
	97		*Tepidimonas taiwanensis strain |1-1 (NR 042418.1)*

[a]The percentage identity refers to percentage of similarity to 16S rDNA sequence.

Reference [12] stated that *Tepidimonas thermarum* was characterized as the strain that do not have the ability to degrade arbutin, casein, elastin, starch and xylan. Similar results observed in this study, where all of the bacterial isolates also showed negative degradation on starch. It has also been reported that, genus *Tepidimonas* was determined to be thermophilic and has the ability to utilize a broad range of carbohydrate including cellulose and carboxymethylcellulose (CMC). The study shows that the strain produced basal cellulase under 55°C with the pH within the range of 7.0 to 7.5. Hence this previous study confirmed that the genus *Tepidimonas* might have strains that can degrade cellulose.

In a study of characterization by [14], it has been reported that the enzyme produced by the strain has the optimum temperature of 50°C to 55°C. The enzyme remained stable within a broad pH range from 7.0 to 10.0. This proved that *Tepidimonas thermarum* strain AA1 is a thermophile and an alkalophilic bacterium.

IV. CONCLUSION

The data gathered in this study provides evidence for the existence of cellulolytic thermophiles in Sg. Klah, Hot Spring. It can be concluded that all the thermophiles that has been successfully isolated derived from the same genus of *Tepidimonas.* The bacterial isolates of M7T55D1, M7T55D2, M7T55D3, M7T53D1, M7T53D2 and M7T53D3 were identified as alkalophilic cellulolytic thermophiles as it has the ability to degrade the carboxymethylcellulose (CMC) at temperatures between 50°C and 55°C with a pH value of above 7.0 and 7.5. This ability may make them an important biotechnological tool for thermostable cellulase production.

The investigations clearly indicate that the Sg. Klah hot spring is a potential source of many thermophilic bacteria and need to be explored for the industrially viable enzymes by further studies on the microbiological aspects and meta-genomics to explore the uncultivated organisms. To further validate the isolates as thermo tolerant cellulase producers, enzymatic assay procedure is recommended.

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