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Journal of Biotechnology Science Research.2016;3(5):166-174

Journal of Biotechnology Science Research (JBSR)

Journal homepage: www.jbsr.issres.net

Original paper

Preliminary Phylogenetic Analysis of Thermophilic Bacteria Producing Protease isolated from Kuantan Malaysia

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ARTICLE INFO:

Article history:
Received: 12 Mach 2016
Received in revised form: 25 April
Accepted: 10 January 2017
Available online: 18 January 2017

Keywords:
Thermophilic bacteria
Protease
PCR
Molecular analysis

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ABSTRACT

Thermophilic bacteria grow in relatively high temperature between 50 to 60°C. These organisms can produce specific enzymes that have been used in industrial and biotechnological applications. The basic method to derive a sequence for a uncultured bacteria is to use universal primers against the 16S rRNA gene region in a PCR step to increase the amount of DNA and then to sequence the amplicon. This study was carried out to screen the ability of thermophilic bacterial isolates for degrading protein by protease and to identify the most potent isolates using molecular techniques inferred by 16S rRNA gene. We are dependent on accurate sequences in databases, appropriate names associated with those sequences, and an accurate sequence for the isolate to be identified. Thirty seven bacterial isolates were cultured and characterized using nutrient casein plate method. Twenty out of thirty seven bacterial isolates have been recognized to be positive for casein test and selected for Gram staining method. Total DNA genomic were extracted by using I-genomic DNA Extraction Mini Kit, and polymerase chain reaction (PCR) operation assay was performed for appropriate target isolates. The phylogenetic analysis of these strains revealed that the genus of Bacillus is the most closely matched to the data from the GenBank. *Bacillus subtilis*, *Bacillus* sp, *Bacillus amyloliquefaciens* and *Bacillus licheniformis* were identified as species for the potential bacterial isolates. Two samples which are RSS6 and JF OUTER 2B have not been characterized through the phylogenetic tree. This might refer to either rare or new bacteria.

1. Introduction

Considered as ubiquitous microorganism, bacteria can survive in all sorts of inhospitable environments. The studies in the last two decades showed that about 99% of bacteria are still unexplored and unexploited regarding ecological functions and biotechnological applications[1,2]. Bacteria are classified into several classes based on their temperature ranges for growth, namely, psychrophiles, mesophiles, thermophiles and hyperthermophiles. Thermophilic bacteria, derived from the Greek term (thermotita, philia) meaning heat and love, respectively, can thrive at relatively high temperature from 45°C to 80°C [3, 4]. Recently, most research related to hot spring microbes have focused on cultivating and isolating of extreme thermophilic and acidophilic strains [5]. Thermophilic organisms have adapted to live in extreme environments due to lipid membrane structure which is saturated by chain fatty acids than others [6]. This property allows thermophiles to grow at higher temperatures by providing the right degree of fluidity required for membrane function. Microorganisms, which live under extremely condition such as hot springs, volcanic and geothermal regions, have unique features that are used for industrial applications. Thus, there have been increasing toward studying thermophilic bacteria by scientists as potential source

for thermostable industrial enzymes [7,8]. Identified as the most significant hydrolytic enzymes, microbial proteases have been studied extensively since the advent of enzymology. There is renewed concern in the study of proteolytic enzymes, especially, for those enzymes that not only play an important role in the cellular metabolic processes but have also procured considerable interest in the industrial community [9]. Proteases are essential components of all forms of life on earth, including prokaryotes, fungi, plants and animals. Proteases are also defined as enzymes that hydrolyze peptide bonds in proteins. They constitute one of the most significant groups of industrial enzymes extensively used in the food, pharmaceutical, protein hydrolysis, detergent, cheese-making, brewing, photography, baking, and leather industries. Moreover, these enzymes have been included in animal and human food as digestive aids[10,11,12]. In the recent years, 16S rRNA gene has been considered as the ‘gold standard’ for probing the species structure of a variety of environmental bacterial communities [13]. The 16S rRNA molecule comprises conserved region and nine variable regions (V1-V9) which provide genetic information to distinguish bacteria up to species and subspecies levels [14]. In this study, the ability of thermophilic bacteria to secrete protease has been reported with identification of these isolates based on molecular technique (16S rRNA).

2. Material and Methods

2.1 Isolation of thermophilic bacteria

In this study, three different sources were used for microbial isolation from soils, water, and food. Two soil samples collected from Panching area (PS) and Kolej Kediaman 2 (KK2S) inside and around Universiti Malaysia Pahang (UMP) area. Two water samples were collected from Sungai Belat (RSS) and Gambang Lake (GL), Pahang, Malaysia. Three food samples were collected from chicken liver (CL), spoilt apple (A) and jack fruit (JF). Serial dilution method was used, dilution (10-1 – 10-5) was streaked onto the surface of Nutrient Casein Medium plates (8 g/L casein and 23 g/L nutrient agar) and incubated at 50°C for 48 hours.

2.2 Identification and characterization of the isolates

Bacterial isolates were streaked on nutrient agar medium and incubated at 50°C for 48 hours. They were transferred and incubated at 50°C for 24- 48 hours into Nutrient Casein medium (8 g/L casein and 23 g/L nutrient agar). After terminating incubation period, the plates were flooded with 5ml of mercuric chloride and incubated inside the chiller for 10 minutes. A zone of proteolysis was examined on the casein agar plates. All bacterial isolates were subjected to Gram staining and then the shape and color of these isolates were recorded.

2.3 Genomic DNA preparation and PCR amplification

Genomic DNA was extracted from the selected isolates which contain positive and negative Gram using G-spin™ Genomic DNA Extraction Kit (iNtRON, Korea), according to the manufacturer's instructions. Each genomic DNA has been used as template for 16S rRNA gene amplification. PCR reaction was done with aid of universal primer 27F 5’-AGAGTTTGATC (A/C) TGGCTCAG-3’ and 1492R 5’-GGTTAC (G/C) TTGTACCTGCCGGA-3’. The PCR mixtures (50 µl) were freshly prepared in a sterile PCR tube. The master mix contains dNTPs (1.0µl), primer forward (2.5µl) and reverse (2.5µl), DNA template (2.0µl), buffer/ MgCL2 (5.0 µl), Taq DNA polymerase (0.2µl) or Pfu DNA polymerase and deionized water was added until the total volume of 50µL. The reaction tubes were placed into PCR machine using a mastercycle (Biorad, USA). PCR products were run on a 1.2% agarose gel to determine

the size of the PCR products. The purity and concentration of DNA was measured using NanoVueTMPlus Spectrophotometer (GE Healthcare). The PCR products were sent to (1st BASE Molecular Biology Services) Company after DNA sequencing had been performed. The 16S rRNA sequence were aligned and compared with the sequences deposited in Gene Bank database from National Center for Biotechnology Information (NCBI), using Blast analysis tool. After that, the command of CULSTAL-W was used in order to align the sequences with others that obtained from the BLAST result page. Next, the construction of phylogenetic tree was allocated by using the neighbor-Joining method (Saitou and Nei, 1987) with bootstrap values by using MEGA software MEGA version 5.2.2. [15].

3. Results and Discussion

3.1 Screening of protease producing thermophilic bacteria

A total of 40 bacterial isolates were cultured on nutrient agar. Thirty seven isolates have successfully grown meanwhile the remaining three isolates, which are A8 and A2B, A2C did not show any sign of growth. The specimens were incubated at 50°C for 48 h. The growth of all isolate was recorded in (Table 1). The researcher had reported that the soil and aquatic sediments from the temperate environments are known to possess thermophiles bacteria [16]. The isolates were screened for producing of protease. Strains were cultured on nutrient agar supplemented with casein. Therefore, the samples with higher protease activity were selected according to the clearance area around the colonies (the proteolysis area based on different isolates). The temperature should be kept at 50°C for confirming that the bacteria had been supplied by suitable temperature in order to remaining alive. Casein hydrolysis was used to observe whether protein is degraded into amino acids or not [17]. Out of the thirty seven isolates, twenty samples were observed to be a protease producer by showing positive protease clearing zone around colonies (Table 1). The clearing zones (Fig.1) represent the proteolytic breakdown of milk proteins and indicate to secreting of protease. The clear zone formed around the colony can be used only to screen protease producing by bacteria [18, 19].

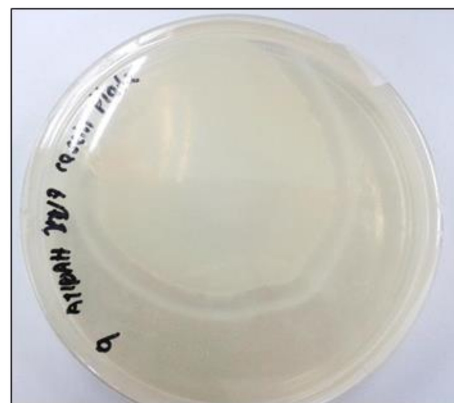

Figure 1 Protein degradation by JFOUTER 2B

3.2 Gram reaction characteristics

Gram reaction results showed that 12 isolates (8 from food, 3 from water, and 1 from soil samples) were Gram positive whereas another 8 isolates (7 from food and 1 from soil samples) were Gram negative (Table 2). All bacterial isolates appeared in long rod shape. According to Hussin et al. (2011) [16], morphological studies, conducted on the thermophilic strains, revealed that bacterial isolates were long and short rod shaped with Gram positive and negative in character. Therefore, all isolates, which fulfilled the characteristic of thermophilic bacteria, were selected to proceed to the next procedure.

Table 1 Screening of protease producing thermophilic bacteria

No	Isolates Code	Isolate Source	Protease clearing zone	Gram reaction and shape
1	RSS5	Water	-	ND*
2	CL4C	Food	+	+ve rod
3	CL16	Food	-	ND
4	JF INNER	Food	-	ND
5	JF OUTER 2A	Food	-	ND
6	RSS2B	Water	-	ND
7	CL8A	Food	+	-ve rod
8	RSS3	Water	-	ND
9	CL13B	Food	+	+ve rod
10	CL3	Food	+	-ve rod
11	GL7	Water	+	+ve rod
12	JF OUTER 1	Food	+	+ve rod
13	CL11	Food	+	-ve rod
14	CLZC	Food	-	ND
15	CL7	Food	-	ND
16	PS1	Soil	+	+ve rod
17	CL4B	Food	-	ND
18	CL5	Food	+	+ve rod
19	KK2S4	Soil	+	-ve rod
20	CL9	Food	-	ND
21	CL2 Branch	Food	+	-ve rod
22	A2A	Food	+	-ve rod
23	GL5C	Water	+	+ve rod
24	RSS6	Water	+	+ve rod
25	KK2S5	Soil	-	ND
26	RSS5	Water	-	ND
27	CL14	Food	+	+ve rod
28	KK2S6A	Soil	-	ND
29	CL2C	Food	+	+ve rod
30	CL2A Branch	Food	+	-ve rod
31	A9	Food	-	ND
32	JF OUTER 2B	Food	+	+ve rod
33	A1	Food	+	-ve rod

34	CL13A	Food	-	ND
35	A5	Food	+	+ve rod
36	JF OUTER 2A1	Food	-	ND
37	A6	Food	-	ND

*(ND): Not detected; (+): Protease positive; (-): Protease negative; (+ve): Gram positive bacteria; (-ve): Gram negative bacteria.

Table 2 Yield and purity of the extracted genomic DNA for selected bacteria

No	Isolates	Gram reaction	A260/A280	Concentration (ng/µl)
1	RSS6	G +ve	1.863	26.0
2	JF OUTER 2B		1.811	77.0
3	GL5C		1.718	30.5
4	GL7		1.683	74.0
5	CL13B		1.890	35.5
6	CL14		1.980	54.5
7	PS1		1.635	42.5
8	CL5		1.663	66.5
9	JF OUTER 1		1.806	102.0
10	CL4C		1.543	71.0
11	A5	G -ve	1.452	106.0
12	CL2C		1.934	70.5
13	CL8A		1.657	54.5
14	A1		1.573	59.0
15	CL3		1.645	76.5
16	CL2A BRANCH		1.631	80.5
17	CL11		1.602	98.5
18	A2A		1.857	58.0
19	CL2 BRANCH		1.554	50.5
20	KK2S4		1.396	21.5

3.2 Molecular identification of the selected thermophilic isolates

The genomic materials of the samples were isolated by using G-spin™ DNA Extraction Kit (iNtRON, Korea) based on the manufacturer's instructions. DNA was detected by gel electrophoresis method. However, only seven isolates (highest purified DNA showed 1.8 and above) were selected for Amplification of 16S rRNA Gene due to the DNA purity (Table2). PCR was carried out by using the extracted bacterial DNA and universal primers. Gel electrophoresis was carried out in order to analyze the outcome of PCR reaction (Fig 2 and 3). Based on Fig 2, lane 2 to 6 and lane 2,3 for Fig 3 exhibited a thick band maybe due to high concentration of 16S rRNA gene with approximate 1500 bp comparing to the DNA ladder in the DNA ladder in the lane 1. DNA sequencing was performed. All of the 16S rRNA genes of seven isolates subjected to Clustal-W software. The conserved region of all isolates range from 533 to 1090 bases. The sequences from all the seven isolates were aligned with their closely related other bacterial sequences obtained from the GenBank by Basic Local Alignment Search Tool (BLAST) program [20]. The sequences were chosen from the result of BLAST to perform the alignment by using MEGA version 5.2.2 software and the Neighbor-joining bootstrap method were used to construct the phylogeny tree as shown in Fig 4.The alignment revealed that sample GL4 (A1), JF OUTER 1 (C1), CL2C (E1) fell within the genus *Bacillus* .The three organisms were finally designated as *bacillus subtilis*, *Bacillus* sp, and *Bacillus amyloliquefaciens*. Isolates CL14 (B1) and CL13B (H1) can be identified as the genus of *bacillus*. Therefor, Current isolates are designated as *Bacillus* sp and *bacillus licheniformis*, respectively. Regarding sample A2A (D1), it was clustered with the *Bacillus sp* or with *Enterobacter cloacae* based on the similarity from the phylogenetic. As shown from the tree, the identification of RSS6 (F1) and JF OUTER 2 (G1) has not been determined for each sample even at genus or species level. These bacteria can be assumed as rare bacteria. Thus, further analyse required for those two samples which might aid to identify the new thermophilic bacteria that can degrade protein and produce the protease enzyme.

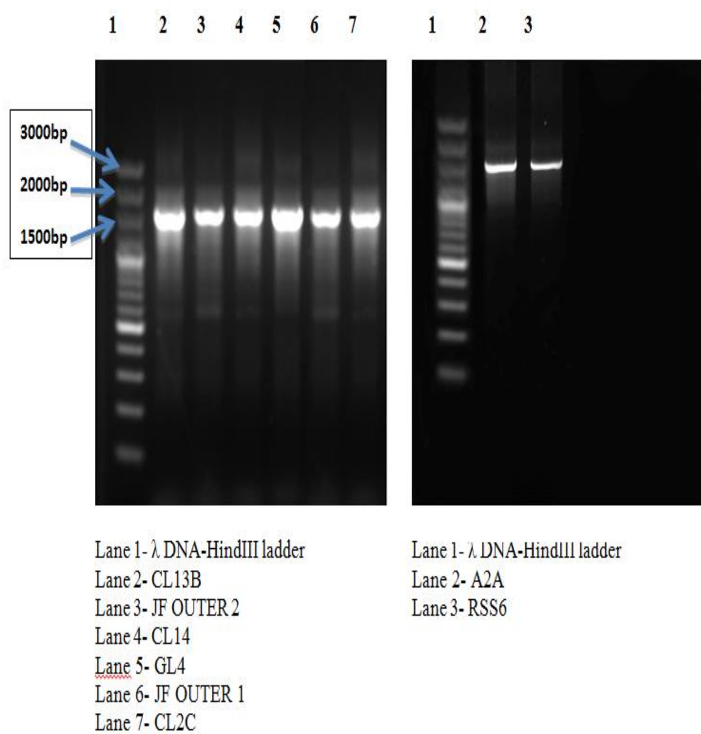

Figure 2 and 3 Amplification of 16s rRNA gene

Figure 4 Phylogentic tree using nighbour-joining method of Thermophilic bacterial strains to other species.

4. Conclusion

Protein was successfully degraded into amino acid by the action of thermophilic bacteria with the aid of protease enzyme. 20 isolates has been reported in this study to produce different halo zones around the colonies which indicate to positive protease activity. The potentially thermophilic bacteria were identified morphologically through Gram staining protocol. All the 20 isolates (12 isolates; 8 from food, 3 from water, and 1 from soil samples are Gram positive whereas another 8 isolates; 7 from food and 1 from soil samples are Gram negative) were described as rod in shape. Twenty isolates were subjected to DNA extraction and seven isolated were selected as the most potent for amplification of 16S rRNA gene and sequencing due to the appropriate purity of DNA. As stated from the phylogenetic tree, samples (A1), JF OUTER 1 (C1), CL2C (E1) CL14 (B1) and CL13B (H1) belonged to Bacillus and designated as *Bacillus subtilis*, *Bacillus sp*, *Bacillus amyloliquefaciens*, *Bacillus sp* and *Bacillus licheniformis*, accordingly. In addition, sample A2A (D1) was clustered with the *Bacillus sp* or with *Enterobacter cloacae* based on the similarity from the phylogenetic. However, RSS6 and JF OUTER 2B have not been recognized through the phylogenetic tree. Thus, further investigations are required to confirm the genus of these isolates

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

Grateful acknowledgements are made to the Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang (UMP) for the financial support using grant # RDU 1403100.

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Cite this article as: Makky EA, Zahid S M Z, AlMatar M, Yusoff M M (2016) Preliminary Phylogenetic Analysis of Thermophilic Bacteria Producing Protease isolated from Kuantan Malaysia. **Journal of Biotechnology Science Research 3(5):166-174**.

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