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CHARACTERIZATION OF *Lysinibacillus* spp. (STRAIN G6) FOR HYDROLYSING PORCINE GELATINE

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ABSTRACT

This study was conducted to identify and characterize gelatinase bacterium of *Lysinibacillus* spp. (Strain G6) for hydrolysing porcine gelatine. The Strain G6 colony was chosen due to its high ability to hydrolyse porcine gelatine when it was grown on medium containing porcine gelatine by forming a clear zone. The bacterium was identified as *Lysinibacillus* spp. Strain G6 using the partial sequence of 16S rDNA analysis with 95% of similarity. Meanwhile, by using Biolog GEN III, the bacterium strain was identified as *Lysinibacillus sphaericus* analysis at 42% of similarity. Gelatinase from the bacterium has been partially purified using deposition of ammonium sulphate and tube dialysis. The partially purified gelatinase of Strain G6 showed significant different ($p<0.05$) in porcine gelatinase activity of $9.12^{a} \pm 2.6$ U/ml followed by bovine ($5.43^{b} \pm 0.8$ U/ml) and fish gelatine ($0.14^{c} \pm 0.7$ U/ml). The molecular weight of gelatinase of Strain G6 was 123.35 kDa. Application of partially purified gelatinase onto porcine, bovine and fish capsules substituted into the gelatin medium (GM), respectively resulted in $11.86^{a} \pm 0.2$ U/ml, $5.39^{b} \pm 2.1$ U/ml and $0.36^{c} \pm 0.2$ U/ml of enzyme activity, respectively. Thus, *Lysinibacillus* spp. Strain G6 bacterium showed greatest gelatinase activity towards porcine gelatine which can be potentially used for porcine gelatine identification.

Key words: Gelatine, gelatinase, gelatinase bacteria, *Lysinibacillus* spp.

INTRODUCTION

Gelatine is a protein product formed by partial hydrolysis of collagen found in bones, hooves, connective tissues, and skin of animals with thermal treatment. It is a hydrocolloid or protein which are unique and serve multiple functions with a wide range of application in various industries including food, beverages, pharmaceuticals and cosmetic (Sahilah *et al*., 2012). Gelatine can be derived from different sources or species of animals such as porcine, bovine, chicken, fish and many more. Different type of species of gelatine will have different physicochemical characteristic and qualities (Karim & Bhat, 2009). Porcine gelatine offered several advantages over bovine or fish due to the cheap, easy, higher volumes yield and takes a shorter time in every cycle of gelatine product. A cycle of porcine gelatine production will take approximately 30 days however, bovine takes at about 60 to 80 days (Sahilah *et al*., 2015).

It is crucial to identify the species of gelatine as it is important for the consumer to know and avoid any allergy of certain sources and also in the religious aspect. Muslims are facing difficulties to ascertain which product is permitted (Halal) or not (non-Halal or Haram) under Islamic law. The various use of porcine gelatine in industries is expanding and the exposure of non-Halal gelatine is not only towards Muslims but also other communities such as Jews, people who are allergic toward hidden porcine ingredients, vegetarians and meat sources in processed foods (Tanabe *et al*., 2007). Other than that, the emergences of Bovine Spongiform Encephalopathy (BSE) or mad cow disease in the 1980s which restricts the use of bovine (Morisson *et al*., 1999; Karim & Bhat, 2009). So it is important to know species of gelatine used in industries to

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avoid any problems for consumers. The alternative gelatine substitute from plant-based such as Arabic gum, seaweeds (carrageenan) and fishes (fish gelatine) was used to fulfill industry but less demand (Karim & Bhat, 2009).

Various types of polymerase chain reaction or PCR is well-known for detection of porcine gelatine and other species of gelatine. Focusing on identifying porcine gelatine in the foods industry, detection used such as conventional PCR, Real Time PCR, species-specific PCR and southern hybridization PCR is commonly used. The gelatine detection through the PCR in foods is well documented (Shabani *et al.,* 2015). Real time PCR technique was developed by many researchers for rapid detection (Demirham *et al.,* 2012; Cai *et al.,* 2012). While the detection of the gelatine differentiation of porcine and bovine has been reported using Fourier transform infrared spectroscopy (FTIR) (Hashim *et al*., 2010).

The potential gelatinase bacteria that can specifically hydrolyse porcine gelatine are important to differentiate species of gelatine from porcine or others. Indeed, previous research was very limited to porcine gelatine detection but the bacteria that specifically hydrolyse porcine gelatine is still questionable. Thus, the objective of this study was to isolate and characterize gelatinase bacteria for hydrolysing of porcine gelatine as one way to determine gelatine species especially porcine gelatine.

MATERIALS AND METHODS

Sample sources

A total of fourteen samples (n=14) were collected soils, skins and water to isolate the gelatinase bacteria. The samples were collected from the area of Endau, Johor in Jan 2014. All samples were stored in sterile conditions at -20°C for further analysis.

Isolation and screening of microorganism

Prior to the analysis, 1.0 g of soil was mixed with 2 mL saline solution (1.5% w/v). The mixture were diluted from 10-1 to 10-3 and spread onto Gelatine medium (GM) agar in duplicates [Gram per litre, g/L medium: 15.0 porcine gelatine (Sigma), 3.0 beef extract (Merck), 5.0 bacteriological peptone (Oxoid) and 15.0 agar (Oxoid), pH adjusted to 7.2] (Su *et al*., 1991). Positive results indicated by halo forming around the grown colonies. All gelatinase bacteria were grown onto nutrient agar slant and kept at 4°C for maintenance. For long preservation, the bacteria were stored in 20% (v/v) glycerol and kept at -20°C.

Screening on gelatine plate medium

The colonies obtained from previous screening were tested for gelatinase activity as described by Pires-Boucas *et al*. (2010). The gelatinolytic activity was tested by inoculating colonies onto nutrient media containing bacteriological agar (1.5% w/v) with 3% (w/v) of porcine; and bacteriological agar (1.5% w/v) with 3% (w/v) fish gelatine, respectively. They were then incubated overnight at 37°C. The gelatinase activity was observed when flooding with mercuric chloride ($HgCl_2$) reagent (Frazier *et al.,*1926) or trichloroacetic acid (TCA) solution (Medina and Barasi, 2007). The enzyme activity was rated by measuring the diameter of the clear zone (R) forming around the bacteria colonies divided by the diameter of the colony (r) (Pires-Boucas *et al*., 2010).

Biolog identification system analysis

Identification of porcine gelatinase bacteria was conducted using Biolog GEN III Identification System (Biolog, USA).

Bacteria growth and DNA extraction

A number of bacteria about 1×10^8 cfu/ml colonies were grown overnight at 37°C on nutrient broth (Oxoid) [g/L; 10 peptones, 10 beef extract, 5 sodium chloride] and 1 mL (w/v) was transferred into microcentrifuge tubes and centrifuged at 10,000 rpm for 5 min. The DNAs were extracted using DNEasy Blood and Tissue kit as instructed by the manufacturer (Qiagen, 2006).

Partial sequence of 16S rDNA analysis

The analysis of 16S rDNA was done using the method described by Lee *et al.* (2009) using universal primers of Forward: 5'-AGAGTTTGAT CMTGGCTCAG-3' and reverse: 5'-AAGGAGGTG WTCCARCC-3'). Expected amplicon was 1500 bp. The amplicon was sent to (OLIPRO, MY) laboratory and 16S rDNA sequenced data were compared in the NCBI (National Center for Biotechnology Information) GenBank databases using BLAST (Basic local alignment search tool) software available at https://www.ncbi.nlm.nih.gov/.

Extraction and purification of enzyme using Ammonium Sulphate Precipitation

A total of 1×10^8 cfu / ml of active colonies of bacteria obtained in subcultures in a nutrient broth overnight at 37°C. Same number of active cells are then transferred into the enzyme accumulation medium containing phosphate buffer 0.04 M K_2HPO_4 and 0.06 M $Na_2HPO_4.7H_2O$ K_2HPO_4 in Ph 7.2 (Mazotto *et al*., 2011) together with 1% (w/v) gelatine as the sole source of nitrogen and carbon and incubated in incubator shaker for two days at

30°C with 170 rpm. Enzyme was then concentrated using Falcon vivaspin tube (Stedim *et al*., 2014) by centrifuging at temperature 20°C with 5,000 g for 10 min.

Then, 1 mL of clear supernatant was added with 30% saturation of ammonium sulphate in the centrifuge tube and let it for 10 min for protein precipitation process. The precipitate was removed by centrifugation at 20,000 g for 15 min and the supernatant was added with 70% ammonium sulphate and let it for 10 min for protein precipitation. Pellet was collected by centrifugation at 20,000 g for 15 min and was dissolved with distilled water. Purification of the enzyme was done by salting out method through the Visking tube dialysis technique as described by (Stedim *et al*., 2014).

Enzyme quantitative for gelatinase activity

The quantitative activity of gelatinase was determined according to Sajitha *et al*. (2011). Enzyme sample (crude and partially extracted) with buffer solution was incubated at 37°C for 30 min. The reaction mixture contained 1% (w/v) gelatine (porcine, bovine or fish) as substrate solutions from commercial gelatine. Enzyme activity was expressed as one unit of gelatinolytic activity as the amount of enzyme required to produce an increase of 0.01 absorbance unit at 280 nm under standard assay condition (30 min at 37°C).

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The partially purified protein stock was prepared as described by Mazotto *et al*. (2011). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the partially purified enzyme was performed using 15% gel slabs and 5% stacking gel using method of Laemmli (1970) and Jeong *et al*. (2001) at 4°C. For sample preparations, samples were reduced by adding 0.5 µL, 1 M of 1,4 dithiothreitol (DTT) to denature the protein and boiled for 10 min. Samples prepared were then loaded into wells of acrylamide gel. Molecular mass standard marker Protein (standard molecular weight PageRulerTM Prestained Protein Ladder ~10-180 kDa (26616) from Thermo Scientific) were used as the standard marker. Electrophoresis was carried out at 100 V for 90 min. The gels were stained with staining solution (0.1% (w/v) Coomassie brilliant blue G250 (Sigma), 10% (v/v) acetic acid, 50% (v/v) methanol and distilled water) for 5 min and destained using destaining solution (10% (v/v) acetic acid, 50% (v/v) methanol and distilled water) overnight or until the band appear before visualized.

Application on porcine capsule

Three (n=3) known hard capsules were purchased from a pharmacy in Selangor, Malaysia in Jan 2015. The hard capsules were Garlic Pearl Ranbaxy's (porcine based gelatine), Extra virgin oil Halagel (Bovine based gelatine) and Omega-3 Nordic Naturals (fish based-gelatine) was prepared by melting 1% (w/w) of each hard capsule in warm water, respectively. The 1.5 Ml melted hard capsule was added into solution medium as mentioned by Mazotto *et al*. (2011) and Jones *et al*. (1998) for detecting porcine, bovine and fish activity in each medium, respectively. The solution contained 100 µL extracted enzyme from the above purification and 900 µL buffer solution. The mixed solution was incubated in temperature 37°C for 30 min. A total of 350 µL from the reaction was added with 500 µL of trichloroacetic acid (TCA) to stop the reaction. The solution was centrifuged at 2500 xg for 15 min, the translucent solution was measured at 280 nm. Consequently, the bovine and fish gelatine were also tested for comparison.

Statistical analysis

Analysis of variance (ANOVA) for gelatine activity was carried out using SPSS program version 21. Means were tested with Least Significance Difference (LSD) test. Statistical different among the treatments was attained at $p < 0.05$ level.

RESULTS AND DISCUSSION

In the present study, a total of 270 bacteria strains were successfully isolated from 14 samples of soils, skins and water. Out of 270 bacteria isolates, a single isolated of bacterial strain G6 obtained the best enzyme activity by the measured diameter of the clear zones formed (R/r) around the colony after 24 h incubation at 37°C using a plate test (Pires-Boucas *et al*., 2010) (Table 1). Table 1 shows the screening of bacterial strain G6 towards porcine, bovine and fish on gelatine medium showed different capability in their gelatinase activity.

The intensity of the enzyme activity of bacterial strain G6 was demonstrated as strong (+++), intermediate (++) and weak (+). Bacterial strain G6 showed the ability to hydrolyse three types of gelatine with different intensity of enzyme activity, porcine gelatine showed strong signal compared to bovine and fish gelatine. The production of gelatinase was detected due to this proteolytic enzyme was hydrolysed gelatine into amino acids. This observation could be seen by adding 10% (w/v) of $HgCl_2$ reagent (Jone *et al.,*

Table 1. Screening of enzyme activity from bacterial strain G6 at different gelatine plate medium

Strains	Source of sample	Gelatinase activity		
		Porcine gelatine	Bovine gelatine	Fish gelatine
G6	Peat soil	++	+	+

Note: +++ = strong enzyme activity, ++ = intermediate enzyme activity, + = weak enzyme activity.

Table 2. Identification of bacterium G6 using Biolog GEN III and 16S rDNA gene sequencing analysis

Strains	Organism type	Identification by Biolog GN species (% Similarity)	Identification by 16S rDNA gene sequencing species (% Similarity)
G6	GP-rodsb	*Lysinibacillus sphaericus* (42%)	*Lysinibacillus* spp. E15 (95%)

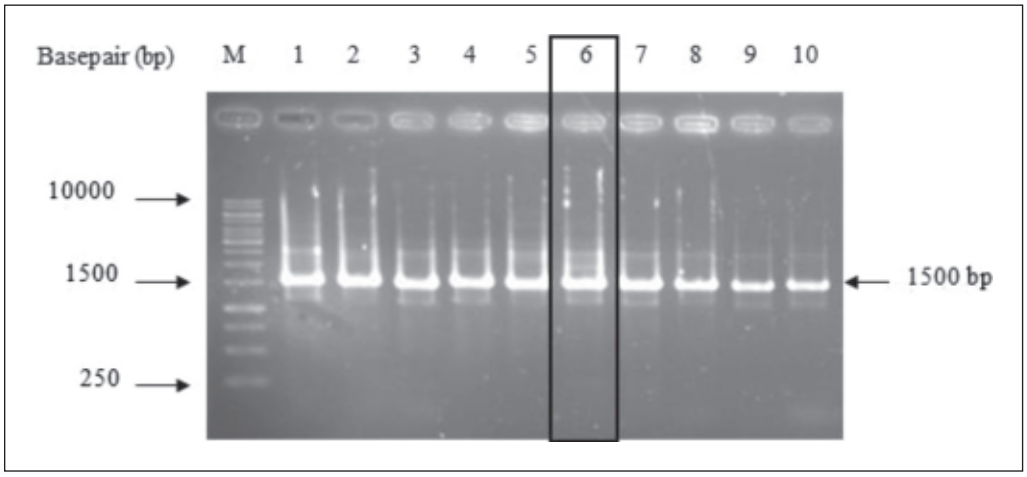

Fig. 1. Amplicon of 16S rDNA fragments of gelatinase bacteria. Lane M: 1kb ladder; Lane 1: Uncultured bacterium clone A16; Lane 2: *Enterobacter* sp. NII-24a; Lane 3: *Bacillus subtilis* strain AB30; Lane 4: *Bacillus cereus* strain JSYM6; Lane 5: *Providencia rettgeri* strain SNCO1_1A; Lane 6: *Lysinibacillus* spp. Strain G6; Lane 7: *Bacillus* sp. enrichment culture clone MJJ-13; Lane 8: *Staphylococcus* sp. NR7; Lane 9: *Pseudomonas optitidis* strain R6-410; Lane 10: Bacterium NLAE-zl-H346.

1998; Frazier, 1926) or TCA solutions (Medina *et al*., 2007) on bacteria colony grew on gelatine plate medium. Our finding was in agreement with Medina *et al*. (2007) who reported, the formation of clear zone around the colony grown on the gelatine medium which indicated amino acids were produced resulting from hydrolysis of gelatine. While white precipitate zone indicated non-amino acids were hydrolysed by gelatinase. Gelatinase is exoenzyme which able to hydrolyse gelatine into free amino acids as a protein source for bacteria.

Bacterial strain G6 was identified using Biolog GEN III and 16S rDNA partial sequence analysis as shown in Table 2 and amplicon was 1500 bp (data are not shown). Table 2 shows the identification of bacteria using 16S rDNA resulted in more than 95% level of similarity rather than Biolog GEN III identification system (42%). Our finding supports the results obtained by Matsui *et al*. (2001) and Morgan *et al.* (2009) who reported the partial 16S rDNA sequences analysis had higher percent accuracy compared to the other methods as described. The 16S rDNA sequence analysis provides high accuracy for identification of any bacterial organism Drancourt *et al*. (2004). Thus, the bacterial was named as *Lysinibacillus* spp. strain G6. As indicated in Figure 1, amplicon of 16S rDNA analysis of gelatinase bacteria (Strain G1-G10) produced 1500 bp using PCR assay. All fragments were purified and sequenced to identify the bacteria species. Sequenced data were analysed with the NCBI (National Center for Biotechnology Information) GenBank databases using BLAST (Basic local alignment search tool) software.

The enzyme extraction and purification were performed in separating biomass. The enzyme was separated and purified through ammonium sulphate precipitation using Visking tube dialysis method.

Table 3. Enzyme activity of partially purified gelatinase enzyme at different gelatine media using absorbance assay at 280 nm

Enzyme sample G6	Gelatinase activity (U/ml)		
	Porcine Gelatine	Bovine Gelatine	Fish Gelatine
	$9.12^{a} \pm 2.6$	$5.43^{b} \pm 0.8$	$0.14^{c} \pm 0.7$

Difference alphabets on the same row show significant difference ($p < 0.05$).

Table 4. Application of pharmaceutical capsules with gelatinase extract from strain G6 sample at absorbance assay 280 nm

Substrate sample	Gelatinase activity (U/ml)		
	Porcine Gelatine	Bovine Gelatine	Fish Gelatine
Pharmaceutical capsules	$11.86^{a} \pm 0.2$	$5.39^{b} \pm 2.1$	$0.36^{c} \pm 0.2$

Difference alphabets on the same row show significant difference ($p < 0.05$).

Ammonium sulphate will bind to molecule gelatinase and increase the molecular weight of gelatinase. As gelatinase has bigger molecular weight than other protein, gelatinase cannot diffuse out from Visking tube. Enzyme activity of gelatinase trapped in Visking tube or called a partially purified enzyme was measured using spectrophotometer at absorbance 280 nm (Mazotto *et al*., 2011). Ammonium sulphate precipitation also used to extract gelatinase from *Brevibacillus laterosporus* conducted by Chi *et al*. (2003). Table 3 shows the enzyme activity of partially purified gelatinase enzyme in *Lysinibacillus* spp. Strain G6. Gelatinase activity measured on porcine gelatine showed a significant ($p< 0.05$) high activity of 9.12 ± 2.6 (U/mL) than bovine and fish gelatine at 5.43 ± 0.8 (U/mL) and 0.41 ± 0.7 (U/mL), respectively. According to result, gelatinase activity of *Lysinibacillus* spp. strain G6 did not show solely specificity towards porcine gelatine due to its abilities to hydrolyse bovine and fish gelatine, at the same time. However, there were significantly different ($p<0.05$) of enzymes activity among porcine, bovine and fish gelatine as a substrate.

The gelatinase molecular weight was determined by sodium dodecyl sulphate-polyacrylamide electrophoresis gel (SDS-PAGE) method. In general, the molecular weight of gelatinase reported more than 10 kDa (Makinen *et al*., 1989; Chi *et al*., 2003; Kanayama *et al*., 2005). However, in this study, our finding showed the gelatinase molecular weight was 123.35 kDa as demonstrated in Figure 2. This result was in contrast with Sandra *et al*. (2013) whose reported two peptide fragments with molecular sizes below 36.8 kDa and 28.6 kDa.

Purified gelatinase was applied to porcine, bovine and fish hard capsules, respectively. As shown in Table 4, *Lysinibacillus* spp. strain G6 has shown the significant ($P<0.05$) highest activity toward porcine capsule at about 11.86 ± 0.2 (U/mL) in value. Those gelatinases also hydrolysed the bovine and fish capsules at the value of 5.39 ± 2.1 (U/mL) and 0.36 ± 0.2, respectively. From our data, there was a significant difference ($p<0.05$) of enzyme activity on the different type of capsules and the result was consistent with enzymes activity as shown in Table 3 and 4. The purification gelatinase from bacterium *Lysinibacillus* spp. strain G6 is not classified as species-specific bacterium toward porcine because it also hydrolysed the bovine and fish gelatine with different degree of enzyme activities. In research conducted by Elgadir

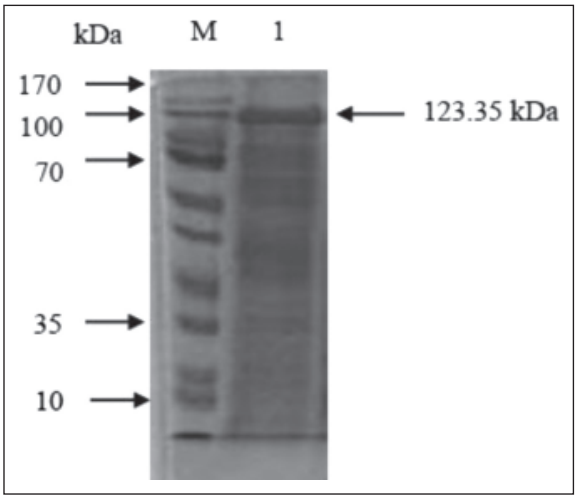

Fig. 2. Electrophoresis was performed using 15% (w/v) SDS-PAGE; Lane M: Molecular mass standard marker Protein (PageRuler^{TM} Prestained Protein Ladder (~10-180 kDa); Lane 1: Size of partially purified gelatinase enzyme from G6 strain (123.35 kDa).

et al. (2013) and Mustafa *et al.* (2013), gelatine from different sources such as porcine, bovine and fish likely have the same amino acid composition. This explained why the gelatinase activity of *Lysinibacillus* spp. strain G6 did not show much different towards three gelatines used as gelatinase cannot act specifically to a specific source of gelatine. In conclusion, in the present study, isolated gelatinase bacteria, *Lysinibacillus* spp. strain G6 was able to hydrolysed porcine gelatine more than bovine and fish gelatine thus showed less specificity of enzyme activity towards porcine. It also indicates *Lysinibacillus* spp. strain G6 may be useful for species identification of porcine, bovine and fish gelatine. The protein purification of *Lysinibacillus* spp. strain G6 which was specific towards porcine will be identified and characterized for future study.

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