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Proteolytic and Lipolytic Activities of *Pseudomonas* spp. Isolated from Raw Milk in Mekong Delta - Vietnam

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Pseudomonas spp. plays an important role in milk spoilage. During the storage of raw milk they produce many thermo-tolerant lipolytic and proteolytic enzymes that reduce both the quality and shelf life of processed milk. This study was performed to isolate, characterize biochemical properties, determine proteolytic and lipolytic activities of *Pseudomonas* spp. and identify the strongest proteolytic and lipolytic activities *Pseudomonas* spp. by 16S rRNA genes sequencing. 14 bacterial isolates belonging to *Pseudomonas* spp. were isolated from 10 raw milk samples from the cow-milk stations and cow-milk farms from Tien Giang, Can Tho, Soc Trang provinces in Mekong Delta, Vietnam. Among 14 isolates, 9 isolates had proteolytic activity, while 10 isolates had lipolytic activity and 8 isolates had both. CT3 isolate had the strongest proteolytic activity and was 100 % of identify with *Pseudomonas putida* strain BASUP8716S rDNA. TG3 isolate had the strongest lipolytic activity and was 100 % of identify with *Pseudomonas putida* strain Seab04 16S rDNA.

1. Introduction

Spoilage of milk resulting from the contamination of dairy products with psychrotrophic microorganisms results in significant losses for the food industry and is a particular concern of the dairy industry (Meng et al., 2017) During the storage of raw milk they produce many thermo-tolerant lipolytic and proteolytic enzymes that reduce both the quality and shelf life of processed milk (El-Roos et al., 2013). *Pseudomonas* has been identified as predominant milk-associated psychrotrophic bacteria, making it one of the most important bacterial groups in the dairy industry. Milk spoilage is caused by the presence of proteolytic enzymes produced by *Pseudomonas* spp. during storage at low temperatures. The most commonly detected *Pseudomonas* species in milk and milk products are *Pseudomonas fluorescens*, *Pseudomonas gessardii*, *Pseudomonas fragi*, and *Pseudomonas lundensis*. *Pseudomonas* spp. can grow over a temperature range of 4 – 42 °C, with an optimal growth temperature above 20 °C (Meng et al., 2017). Although pasteurization of milk has been practiced as the most effective method of reducing the risk of contamination and spreading of disease, many of these enzymes can survive pasteurization (72 °C for 15 s) and even ultra-high temperature treatments (138 °C for 2 s or 149 °C for 10 s) and can thus reduce the sensory quality and shelf life of processed fluid milk products. Post pasteurization contamination contributes most of the microorganisms, primarily *Pseudomonas* spp., that cause spoilage of conventionally pasteurized milk during refrigerated storage (El-Roos et al., 2013).

Vietnam lies in the area of highest economic and milk consumption growth in the world. Vietnam also increased milk yield reached the second highest in Asia with output milk consumption is increasing rapidly (Khoi and Dung, 2014). There is no information about the presence of proteolytic and lypolytic enzymes produced by *Pseudomonas* spp. in raw milk in Mekong Delta, Vietnam for sanitary conditions of processing equipment control, milk processing, milk quality control.

The aim of this study was to determine proteolitic and lipolytic activity of *Pseudomonas* spp. isolated from raw milk samples from cow-milk stations and cow-milk farms and identify the isolates had strongest activities by 16S rRNA genes sequencing.

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2. Materials and methods

2.1 Isolation of Pseudomonas spp

10 samples of raw milk were collected randomly from the cow-milk stations and the cow-milk farms of in Mekong Delta, Vietnam, including 03 samples of Tien Giang province (01 sample form cow-milk farms and 02 samples from cow-milk stations), 04 samples of Can Tho province (02 sample form cow-milk farms and 02 samples from cow-milk stations) and 03 samples of Soc Trang province (01 sample form cow-milk farms and 02 samples from cow-milk stations). The samples were kept in sterilized plastic box, labelled before transferred to laboratory, stored in refrigerator at 4 -10 °C for 24 h for isolation of *Pseudomonas* spp. on Cetrimide-Aga (CA) media (Flint and Harley, 1996) in University laboratory. The raw milk samples were plated initially on CA media and incubated at 37 °C for 24 – 48 h; cultures were streaked on media to obtain single colonies as described by Luong et al. (2013).

2.2 Colony characteristic and microscopic examination

The morphological characterisation of the bacterial colonies were carried out according to on the basis of their shape, size, colour, margin, elevation on the media. Cell morphologies of the isolates were observed using optical microscopes (Olympus DP12 Microscope 100x) (Luong et al., 2003).

2.3 Biochemical tests

The representative suspected colonies were purified and then tested for morphology, motility, Gram stain, Catalase, Oxidase, Citrate utilization, Indole production, Gelatin hydrolysis, Nitrate reduction. The pure isolates were subcultured onto CA agar plates and incubated at 37 °C for 48 h prior to testing. Biochemical tests were carried out according to the methods of Luong et al. (2003).

2.4 Determination of the proteolytic and lipolytic activities of *Pseudomonas* spp.

The putative bacterial isolates belonging to *Pseudomonas* spp. isolated were investigated for their proteolytic and lipolytic activities. To evaluate the proteolytic activity, the isolates were plated on milk agar. The milk agar was prepared with plate count agar supplemented with 1 % skimmed milk powder. The plates were incubated at 37 °C for 4 d. The presence of transparent zones around the spots was recorded as positive strains referring to protease production (Luong et al., 2003). For the evaluation of lipolytic activity, the isolates were plated on tributyrin agar, prepared with plate count agar supplemented with 0.5 % tributyrin. The plates were incubated at 37 °C for 7 d. Lipolytic activity was determined by measuring clear zone around each colony (halo) (Montanhini et al., 2013).

2.5 Identification of *Pseudomonas* isolates

Among proteolytic and lipolytic activities isolates, the strongest proteolytic and lipolytic activities isolates were chosen to sequence and the results were compared to sequences of GenBank based on partial 16S rRNA sequences to show relationships between strains in Nam Khoa company, Vietnam. Bacterial DNA was isolated following published protocols according to Neumann et al. (1992). The amplification of the gene from *Pseudomonas* spp. isolates was performed using 8F (5' - AGA GTT TGA TCC TGG CTC AG – 3') and 1492R (5'-TAC GGT TAC CTT GTT ACG ACT - 3') primers according to Khan and Doty (2009). Partial 16S rRNA gene of selectived isolates in each group were sequenced by MACROGEN, Republic of Korea. Finally, 16S rRNA sequence of the isolate was compared with that of other microorganisms by using the Basic Local Alignment Search Tool (BLAST),with a similarity cut-off of 99%.

3. Results and discussion

3.1 Bacteria isolation, colony characteristic and microscopic examination

14 putative bacterial isolates belonging to *Pseudomonas* spp. were isolated from the 10 raw milk samples (as mentioned above). Among them, there were 4 isolates from Tien Giang (TG1, TG2, TG3, TG4); 5 isolates from Can Tho (CT1, CT2, CT3, CT4, CT5) and 5 isolates from Soc Trang (ST1, ST2, ST3, ST4, ST5) (Table 1). The 14 isolates were spread on the CA media and incubated for 48 h at 37 °C before identification and biochemical test. The results showed that almost their colonies have round-shaped; milky white or opaque white; entire or lobate margin; raised or flat elevation (Figure 1a, 1b, 1c, 1d). Colony diameter ranged from 1 to 2 mm after culturing on CA media at 37 °C for 48 h.

Table 1: Colonies and cells morphology of isolated bacteria

Name of bacterial isolates	Colonies morphology					Cell morphology
	Form	Color	Margin	Elevation	Dimension (mm)	
TG1	Circular	milky white	Entire	Raised	1.0 - 2.0	Short rod-shaped
TG2	Circular	Opaque white	Lobate	Flat	1.5 - 2.0	Short rod-shaped
TG3	Circular	milky white	Lobate	Flat	1.0 - 2.0	Short rod-shaped
TG4	Circular	milky white	Lobate	Flat	1.0 - 1.5	Short rod-shaped
CT1	Circular	milky white	Entire	Raised	1.0 - 1.5	Short rod-shaped
CT2	Circular	milky white	Entire	Raised	1.0 - 1.5	Short rod-shaped
CT3	Circular	milky white	Entire	Flat	1.0 - 1.5	Short rod-shaped
CT4	Circular	Opaque white	Entire	Raised	1.5 - 2.0	Short rod-shaped
CT5	Circular	Opaque white	Entire	Flat	1.0 - 1.5	Short rod-shaped
ST1	Circular	Opaque white	Entire	Flat	1.0 - 1.5	Short rod-shaped
ST2	Circular	Opaque white	Entire	Raised	1.5 - 2.0	Short rod-shaped
ST3	Circular	Opaque white	Lobate	Flat	1.5 - 2.0	Short rod-shaped
ST4	Circular	Opaque white	Entire	Raised	1.5 - 2.0	Short rod-shaped
ST5	Circular	Opaque white	Lobate	Raised	1.0 - 2.0	Short rod-shaped

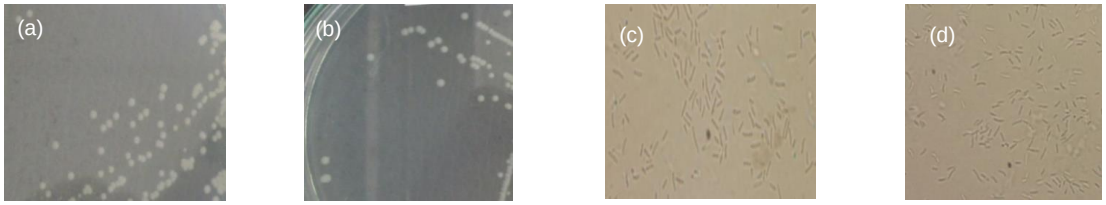

Figure 1: Shape of bacterial colonies on the CA agar after 48 h for (a) TG4 isolate, (b) CT2 isolate); Morphology of isolated bacterial strains in Olympus DP12 Microscope (100x) for (c) ST1 isolate (d) TG3 isolate

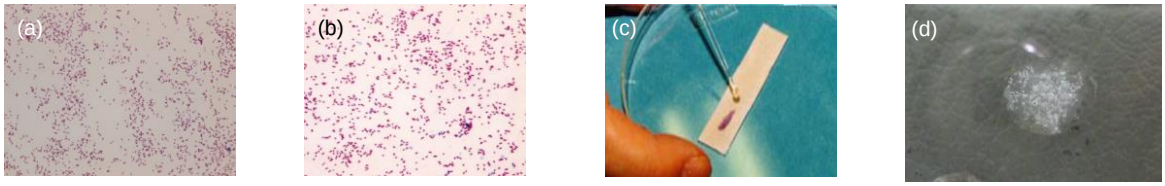

Figure 2: Gram staining of bacterial cells for (a) CT3 isolate, (b) TG3 isolate); (c) Oxidase test (CT3 isolate); (d) Catalase test (CT5 isolate)

All the isolates were Gram-negative, catalase and oxidase positive rods (Figure 2a, 2b, 2c, 2d), utilized citrate, produced indole production, hydrolyzed gelatin hydrolysis and reduced nitrate (Table 2). These characteristics are consistent with previous study of Samet-Bali et al. (2013) in which most *Pseudomonas* spp. bacteria isolated from thirty pasteurized milk samples collected aseptically from different dairy shops, supermarkets and groceries in different areas in Sfax Governorate, Tunisia had the biological characteristics including Catalase (+), Oxidase (+), Citrate utilization (+), Indole production(-), Gelatin hydrolysis (+), Nitrate reduction (+). On the basis, it can be concluded the isolates belong to *Pseudomonas* species.
The results of the phenotypic characterization, based on morphological and biochemical tests, allowed all the isolates were confirmed as *Pseudomonas* species.

3.2 The proteolytic and lipolytic activities of Pseudomonas spp

The 14 isolates were analyzed for the production of proteolytic and lypolytic enzymes. The results were shown in Table 2. Proteolytic activity on the milk agar was determined by the presence of transparent zones around the spots in the inoculated area. After incubation for 4 d at 37°C on the milk agar, proteolytic activity was detected in 64.3 % (9/14) of the isolates (Figure 3a). CT3 generated the largest transparent zone (9.0 mm), indicating strongest proteolytic activity. The CT3 was therefore selected for further study.

The halo was used as an indication to detect the bacterial activity for degrading lipids and producing lipase enzymes. Among 14 isolates, 11 isolates (78.6 %) had lipid-degrading activity (Figure 3b), TG3 generated the largest halo (+++), indicating strongest lypolytic activity. The TG3 was therefore selected for further study.

Table 2: Biological characteristics, proteolytic and lipolytic activities of 14 bacterial isolates isolated from raw milk samples in Mekong Delta, Vietnam

Name of bacterial isolates	Gram (+/-)	Catalase	Oxidase	Gelatin	Citrate	Nitrate	Indol	Proteolytic activity (D/d)	Lipolytic activity
TG1	-	+	+	+	+	+	-	4.5	++
TG2	-	+	+	+	+	+	-	0.0	-
TG3	-	+	+	+	+	+	-	0.0	+++
TG4	-	+	+	+	+	+	-	5.0	++
CT1	-	+	+	+	+	+	-	0.0	+
CT2	-	+	+	+	+	+	-	7.0	+
CT3	-	+	+	+	+	+	-	9.0	+
CT4	-	+	+	+	+	+	-	2.0	+
CT5	-	+	+	+	+	+	-	0.0	+
ST1	-	+	+	+	+	+	-	7.0	++
ST2	-	+	+	+	+	+	-	3.5	+
ST3	-	+	+	+	+	+	-	4.0	++
ST4	-	+	+	+	+	+	-	0.0	-
ST5	-	+	+	+	+	+	-	3.5	-

Gram, Catalase, Oxidase, Gelatin, Citrate, Nitrate, Indol: (-): negative; (+): positive
Proteolytic activity (D/d): d: diameter of colony (mm); D (mm): diameter of zone of around the colony; incubated at 37 °C for 4 d
Lipolytic activity: "+" Zone of 0-1 mm around the colony, "++" zone of 1-2 mm; "+++" zone of >2 mm; incubated at 37 °C for 7 d

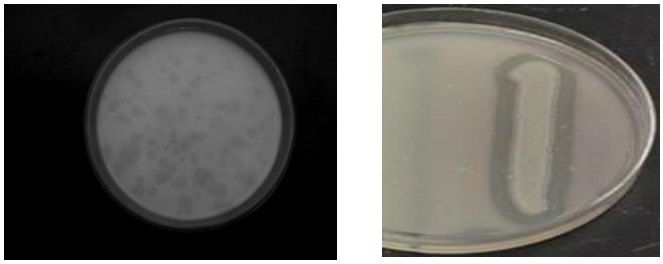

Figure 3: (a) Proteolysis of the CT3 isolate in skim milk agar plate; (b) Lipolytic activity of the TG3 isolate in nutrient agar medium containing tributyri

Psychrotrophics were implicated in many defects in milk and dairy products which are a problem resulting from prolonged refrigeration storage and distribution of perishable food products. The high spoilage potential of *Pseudomonas* spp. is not only because of its ability to multiply at refrigeration temperatures but also because of their ability to produce thermostable proteases and lipases (Omar et al., 2015). *Pseudomonas* spp. produces a large number of extracellular toxins, which include phytotoxic factor, pigments, hydrocyanic acid, proteolytic enzymes, phospholipase and enterotoxins. Exotoxins are responsible for *Pseudomonas* spp. pathogenicity because it can produce leucopoenia, circulatory collapse, necrosis of liver, pulmonary edema, hemorrhage and kidney tubular necrosis. The enterotoxin produced is responsible for diarrhea disease. Generally, they are considered incapable of surviving pasteurization and they may be post-processing contamination (Samet-Bali et al., 2013).

3.3 Identification of the strongest proteolytic and lipolytic activities *Pseudomonas* spp. by 16S rRNA genes sequencing

Based on the strongest proteolytic and lipolytic activities *Pseudomonas* spp. (Table 2), 2 isolates were chosen to identify from 2 provinces (TienGiang – TG3, CanTho – CT3). These isolates were identified by 16S rDNA sequencing, which is a widely used method for the identification of isolates. The 16S rDNA gene was PCR

amplified using universal primers and was sequenced by forward primer. The sequence was matched with NCBI database by BLAST N software. Results of DNA sequencing were presented in Figure 4 (the CT3) and Figure 5 (the TG3). The CT3 was similarity of 100 % with *Pseudomonas putida* strain BASUP8716S rDNA (GU396283.1). The TG3 was similarity of 100 % with *Pseudomonas putida* strain Seab04 16S rDNA (JN630852.1) (Figure 6).

TGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGACGGGAGC
TTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAAC
GTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCG
CTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTA
ACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTC
GGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCGA
CAGAATAAGCACCGGCTAACTCTGTGCCAG

Figure 4: The sequence of the 16S rRNA gene of CT3 isolate

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGAT
GACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTG
GGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCG
GGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCG
ACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAA
GAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTG
ACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

Figure 5: The sequence of the 16S rRNA gene of TG3 isolate

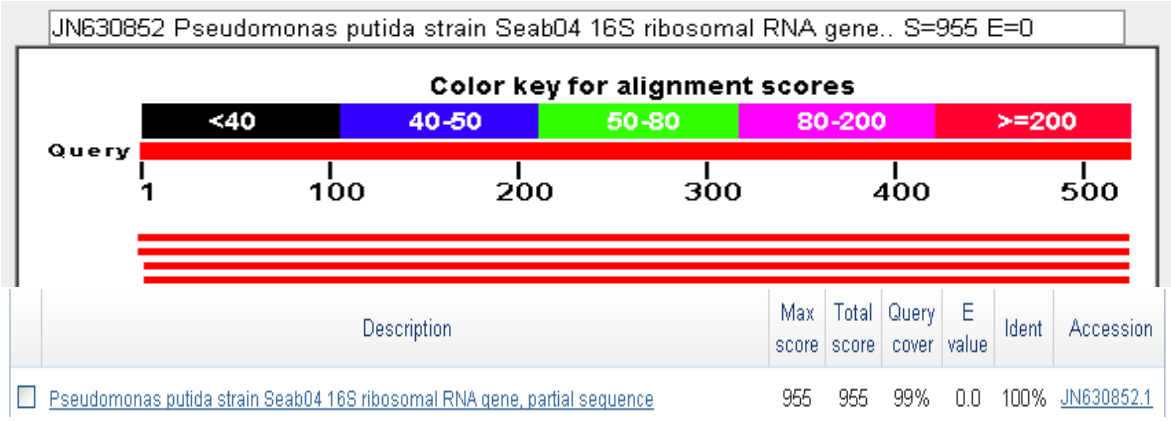

Figure 6: Sequence similarity of the TG3 isolate was compared with strains in GenBank database

Pseudomonas strains were examined for the production of lipolytic enzymes. Positive lipase activity were found in *Pseudomonas putida* and in the strains *Pseudomonas* sp. (Stoyanova et al., 2012). *Pseudomonas putida* grew and formed biofilm at a constant temperature of 5 °C, 10 °C, 20 °C or 30 °C under rich and poor nutrient conditions (Wongsirichot et al., 2019). Subsequently, under the rich nutrient condition, the biofilm detached after it reached maturity at a high temperature, but those at a low temperature remained attached. In contrast, under the poor nutrient condition, biofilm detachment occurred regardless of the temperature condition; thus, lack of nutrients may cause biofilm detachment. Therefore, aside from cleaning blots and strict maintenance of a low temperature during the distribution of agricultural produce, attention must be paid to biofilm detachment as it can lead to an increased risk of bacterial contamination and food poisoning (Morimatsu et al., 2012).

4. Conclusions

Among 14 isolates, nine isolates had proteolytic activity, while ten isolates had lipolytic activity and eight isolates had both. The CT3 isolate had the strongest proteolytic activity and was 100 % of identify with

Pseudomonas putida strain Seab04 16S rDNA. The TG3 isolate had the strongest lipolytic activity and was 100 % of identify with *Pseudomonas putida* strain BASUP8716S rDNA. The future research could be conducted to examine the contamination reasons of Pseudomonas putida into milk and find out the control management to avoid these critical points.

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