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Identification of Biofilm Formation Bacterial Strains Isolated from Raw Milk in BenTre Province - Vietnam

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The presence of biofilms in milk industry may be lead to deterioration in quality of dairy products and a risk to consumer health. The main purpose of the article is to evaluate biofilm formation ability in the 96-well microtiter plate of bacterial strains isolated from raw milk samples of cow-milk farms in BenTre province, Mekong Delta - Vietnam and identify isolates as strong biofilm formers. A total of 10 bacterial isolates is isolated from 8 raw milk samples and studied for their capabilities to form biofilm. All the isolates had biofilm-forming ability. 3, 1 and 6 of them can be classified as strong, moderate and weak biofilm producers. Among the strong biofilm formers, the BT17, BT119 isolates were 100%, 98% of identity *Pseudomonas oryzihabitants* strain 2.1, *Serratia marcescens* NPK2 2 20 respectively. The BT31 isolate had the strongest biofilm ability, extracellular proteolytic and lipolytic enzymes activities and was *Aeromonas allosaccharophila* BT31.

1. Introduction

In the food industry, the presence of bacterial biofilms is a major problem. Biofilms can be characterized as a microorganisms community attached to a surface and enveloped by an array of extracellular polymeric substances aggregated in a moist environment; can settle in the vessels surface, pipes, thermoelectric cooling systems (Silva et al., 2018).

In the dairy industry, the formation of bacterial biofilms is a big risk because of the presence of a variety of microorganisms in raw milk leading to microbial spoilage and to deterioration of quality. Secretion of heat-resistant spoilage enzymes such as proteases and lipases by the biofilm microorganisms into raw milk can lead to reduced shelf life of ultra-high temperature milk (Weber et al., 2019). Some genera *Pseudomonas*, *Aeromonas*, *Serratia*, *Acinetobacter*, *Achromobacter* and *Enterobacter* with predominance of the genus *Pseudomonas* are mentioned as the most frequent representatives of the Gram-negative population isolated from raw milk (Samaržijaet al., 2012). *Pseudomonas* spp. are one of the most important bacteria causing spoilage of conventional raw milk, pasteurized liquid milk products because they produce extracellular lipolytic and proteolytic enzymes in raw milk, pasteurized liquid milk products during storage time even in cooling environments (Simões et al., 2010).

Vietnam was a country having the highest dairy products consumption growth in the world. The consumption are foreseen to increase at 15% and 7% compounded annual growth rate respectively. At the moment, to benefit from the trend, companies in the dairy sector are reshaping their strategies and focusing investments on dairy farming development to increase domestic raw milk supply and to reduce reliance on imports (EVBN, 2016). However, far too little attention has been paid to presence of bacterial strains producing biofilm, extracellular proteolytic and lypolytic enzymes in raw milk in BenTre province, Vietnam for sanitary conditions of processing equipment and poor quality control of raw milk from cow – farmers. There are some methods for detection bacterial communities of raw milk such as culture dependent and independent methods as well as by a combination of both. For direct molecular method, DNA extraction and application of PCR were applied to detect bacteria.

The aim of this study was to assay biofilm formation abilities of bacterial strains isolated from raw milk samples of cow-milk farms and identify the isolates had strong biofilm forming ability by 16S rRNA genes sequencing.

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2. Materials and methods

2.1 Isolation of bacterial isolates and colony characteristic and microscopic examination

A total of 8 raw milk samples were collected randomly from the cow-milk farms of in BenTre province, Vietnam. The samples were kept in sterilized plastic box, labelled before transfer to laboratory, stored in refrigerator at 4 -10 °C for 24 h for isolation in Cetrimide agar (CA: 45.3 g/L Cetrimide agar, 10 ml/L Glycerol; 3.4 g/L Agar) media (Flint and Harley, 1996) in CanTho University laboratory. The raw milk samples were plated initially on the CA media and incubated at 37 °C for 24 – 48 h; cultures were streaked on the media to obtain single colonies as described by Luong et al. (2013). The pure isolates were subcultured onto the CA agar plates and incubated at 37 °C for 48 hours prior to testing. Morphological characterization of the bacterial colonies were carried out according to on the basis of their shape, size, color, margin, elevation on the media. Cell morphologies of the isolates were observed using optical microscopes (Olympus BX51 Microscope 100x) (Luong et al., 2003).

2.2 Biofilm formation assay of bacterial isolates

The method for assessment of biofilm formation of bacterial isolates in the 96 - well microtiter plate was based on the techniques described by Stepanovic et al. (2007). The optical density (OD) measured at 570 nm of each strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the mean absorbance of negative controls (without bacteria) (ODc). The ODc value was calculated by the formula: $OD_C = OD_{Control} + 3*SD$ ($OD_{Control}$: the mean OD of the negative control; SD: standard deviation). The following classification was used for the determination of biofilm formation: no biofilm formation ($OD_{Sample} \leq ODc$), weak biofilm formation ($ODc < OD_{Sample} \leq 2ODc$), moderate biofilm formation ($2ODc < OD_{Sample} \leq 4ODc$) and strong biofilm film ($4ODc < OD_{Sample}$).
The LSD (least significance difference) intervals ($p < 0.05$) were calculated by Excel Software 2010 to evaluate significantly different among treatments.

2.3 Identification of strong biofilm producing isolates by 16S rRNA genes sequencing

Among biofilm formers, strong biofilm formation isolates were chosen to sequence. The results were compared to sequences of GenBank based on partial 16S rRNA sequences to show relationships between strains. Bacterial DNA was extracted from a bacterial suspension (1 ml from a TSB medium at 30 ^{0}C and 120 rpm for 24h) to DNA following published protocols according to Neumann et al. (1992). The amplification of the gene from isolates was performed using 27F (5'-AGAGTTTGATCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') primers according to Frank et al. (2008). Partial 16S rRNA gene of selective isolates in each nitrogen group were sequenced by MACROGEN, Republic of Korea (dna.macrogen.com). 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 98%.

2.3 Biochemical tests, determination of extracellular enzymes activities of the strongest biofilm former

The pure isolates were subcultured onto the CA agar plates and incubated at 37 °C for 48 h prior to testing Gam stain, Catalase, Oxidase, Citrate utilization, Gelatine hydrolysis, Carbohydrate Utilization (Glucose, Lactose and arabinose). Different biochemical tests were carried out according to the methods of Luong et al. (2003).
Determination of protease enzyme activity: The pure isolate was plated on Skim Milk medium Agar (100 g/L skim milk; 5 g/L peptone; 5 g/L NaCl; 3 g/L Yearst Extract; 15 g/L agar) and then incubated at 37 ^{0}C for 72 hours. For the evaluation of lipolytic activity: the pure isolate was plated on Tween 80 hydrolysis media (10 g/L peptone; 5 g/L NaCl; 0,1 g/L $CaCl_2.2H_2O$; 20 g/L agar; 10 ml/L Tween 80) (Kumar et al., 2012). The plates were incubated at 37 ^{0}C for 72 hours. The presence of transparent zones around the spots was recorded as positive strains referring to extracellular protease and lipase production (Luong et al., 2003).

3. Results and discussion

3.1 Bacteria isolation, colony characteristic and microscopic examination

In this study, a total of 10 bacterial isolates were isolated from the samples of raw milk of cow-milk farmers. The details of the colonies and cell characteristics of all 10 isolates were shown in Table 1. All of the colonies were circular; size ranges from 0.5 - 3.5 mm. Color were opaque white, clear white, white, yellow, red; entire or lobate margin; raised or flat elevation (Figure 1). Morphological characteristics of the cells in Olympus BX51 Microscope (100x) (Japan) were short rod-shaped and circular (Table 1).

Figure 1: Shape of bacterial colonies on the CA agar after 48 h (BT17 isolate, (b) BT31isolate); Morphology of isolated bacterial strains in Olympus BX51 Microscope (100x) (c) BT31 isolate, (d) BT119 isolate)

Table 1: Colonies and cells morphology of 10 bacterial isolates

Bacterial isolates	Colonies morphology					Cell morphology
	Form	Color	Margin	Elevation	Dimension (mm)	
BT11	Circular	Yellow	Lobate	Raised	1.2 - 2.5	Short rod-shaped
BT119	Circular	Opaque white	Entire	Raised	0.5 - 2.0	Short rod-shaped
BT16	Circular	Opaque white	Lobate	Raised	1.7 - 2.8	Circular
BT17	Circular	Opaque white	Entire	Raised	2.1 - 3.5	Short rod-shaped
BT31	Circular	Opaque white	Entire	Raised	1.6 - 2.0	Short rod-shaped
BT410	Circular	Opaque white	Entire	Flat	1.0 - 2.0	Short rod-shaped
BT419	Circular	Red	Entire	Raised	1.5 - 2.1	Short rod-shaped
BT42	Circular	Opaque white	Entire	Flat	0.8 - 1.6	Short rod-shaped
BT47	Circular	Clear white	Entire	Flat	0.5 - 1.6	Short rod-shaped
BT49	Circular	White	Entire	Raised	1.6 - 2.0	Short rod-shaped

3.2 Biofilm formation ability of bacteria isolates

A total of 10 isolates were evaluated biofilm formation capacities in the 96 - well microtiter plate at 37 ^{0}C for 48 h. The OD values of the bacteria strains measured at 570 nm were presented in Table 2. The ODc value was 0.11. Results of the classification indicated that all of them had the biofilm formation capacities which 3, 1 and 6 of them strong, moderate and weak biofilm producers respectively. The strong isolates included BT17, BT119 and BT31 isolates. The BT31 isolate had the highest OD value (0.65) and was statistical significant differences with others ($p < 0.05$) which means that this isolate was the strongest biofilm former. The BM16 isolate had the lowest average OD value (0.13). So it could be concluded that the BT31 isolate had the lowest biofilm formation capacity.

Table 2: Optical density (OD) measured at 570 nm of each bacterial isolates

Bacterial isolates	OD_{570} (mean ± Standard Deviation)
Negative control	$0.08±0.01^{g}$
BT11	$0.18±0.04^{de}$
BT119	$\mathbf{0.46±0.03^{b}}$
BT16	$0.13±0.02^{ef}$
BT17	$\mathbf{0.51±0.04^{b}}$
BT31	$\mathbf{0.65±0.01^{a}}$
BT410	$0.20±0.05^{d}$
BT419	$0.14±0.00^{ef}$
BT42	$0.32±0.02^{c}$
BT47	$0.18±0.03^{de}$
BT49	$0.21±0.00^{d}$

$LSD_{0.05}$ = 0.0492; CV = 10.45%

Means followed by different letters in a column are significantly different at $p < 0.05$

3.3 Identification of isolates as strong biofilm formers by sequencing 16S rRNA

The sequences of the 16S rRNA gene of three isolates as strong biofilm formers were presented in Figure 2, Figure 3 and Figure 4. Results of homology search of 16S rRNA gene sequence of selected isolate in GenBank by BLAST were presented in table 3. The results showed that The BT17 isolate was 100%, 98.90% of identity *Pseudomonas oryzihabitants* strain 2.1 (KY623077.1), *Pseudomonas* sp. ZL03

(KJ948663.1) respectively. The BT119 isolate was 98% of identity *Serratia marcescens* NPK2 2 20 (MN691675.1). The BT31 isolate were 100%, 100%, 100%, 98.95%, 98.94%, 98.92% of identity *Aeromonas veronii* strain PCG1 (MN691675.1), *Aeromonas sorbia* strain 109 (MT730012.1), *Aeromonas caviae* strain zj1 (MF445125.1), *Aeromonas veronii* strain JX16102 (KY767538.1), *Aeromonas veronii* strain BL16104 (KY767536.1), *Aeromonas allosaccharophila* strain 78-a blue (MN208205.1) respectively.

The most frequently genera found in milk samples belonged to *Pseudomonas*, *Proteus*, *Psychrobacter*, *Halomonas* and *Serratia*. *Pseudomonas* strains were present in raw milk and produced a large number of extracellular toxins such as enterotoxins causing the diarrhoea disease and producing proteolytic enzymes damaging milk. Milk spoilage is caused by the presence of proteolytic enzymes produced by *Pseudomonas* spp. during storage at low temperature. *Pseudomonas putida* had proteolytic and lypolytic activities and formed biofilm at a constant temperature of 5 ^{0}C, 10 ^{0}C, 20 ^{0}C or 30 ^{0}C under rich and poor nutrient conditions (Le et al., 2020). *Pseudomonas oryzihabitants* is Gram-negative, non-spore-forming, rod-shaped, and is isolated from roots of plants, animals and has plant growth-promoting activities such as silicate solubilization (Fitriyanti et al., 2017).

GGGGACTTGGCGCGGCTACACATGCAGTCGAGCGGATGAGAGGAGCTTGCTCCTCGATTCAGCGGCGGACGGGTGAGTAA
TGCCTAGGAATCTGCCTAGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGTGG
GGGATCTTCGGACCTCACGCTATTAGATGAGCCTAGGTCGGATTAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGACGA
TCGGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG
AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTCGTAAAGCACTTTAAGTT
GGGAGGAAGGGCTCATAGCGAATACCTGTGAGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGC
CGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTTGATAAGTTGGATGTG
AAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGTCTGGCTAGAGTGCGGTAGAGGGTAGTGGAATTTCCAGTGTA
GCGGTGAAATGCGTAGATATTGGAAGGAACACCAGTGGCGAAGGCGACTACCTGGACTGACACTGACACTGAGGTGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGATCCTTGAGATC
TTAGTGGCGCAGCTTACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCA
GAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGTAACGAGCGCAACCTTGTCCTTAGTTCCAGCACGTTATGGTGGGCCTCTAGGAGACTGCCGGGACAACCGGAGGA
AGGGGGGGATGACGTCAAGTATCATGGGCCTTCGGCCGGGGTACACCGGTTTAATGTGGGTAAAAGGGTGCCAGCCGCGG
GGGGGGTATCCCTAAAACAAACGAATCCGGATCCAATTGCACTCCCTGCGGGAGAGGAATCCTTGTATTTGAAAAAAAAAA
AGGAAAAATTCCGGGTTTACCCCCAAAAA

Figure 2: The sequence of the 16S rRNA gene of BT17 isolate

GGGCAGTGGGGGCAGCTACACATGCAGTCGAGCGGTAGCACAGGGGAGCTTGCTCCCTGGGTGACGAGCGG
CGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGC
ATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAG
TAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTG
AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCA
TGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAACTTAATACG
TTCATCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGT
GCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGG
GCTCAACCTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGC
GGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGG
TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAAGGT
TGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAA
AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACC
TTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGCATTGGTGCCTTCGGGAACTCTAAGACAGTGCTGCAT
GGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCACGAGCGCAACCCTTATCCTTTGTTGCCGC
GGTTCGGCCGGGAACTCAAAGGAGACTGCCCTGATAACTGGAGAAAGGGGGGGGATGACGTCCAGTCATCATG
GCGCTTCCAATAAGGCTACCACCGGCTCCATGGCGTAACAAAGAAAAGAACCTCCCCCAACAAGCGGACTCCC
AAAAAAGCCAAACCCCGATGGAGGGGTGCCCCCCCCCCCCTAAGCGCGAACCCTTTTTTTTTAAAATAAAAAG
TTCGATAAATAAAAATTTCCCCCCCCTTCTGCCCCCCCCCCCCCCCCCCGCG

Figure 3: The sequence of the 16S rRNA gene of BT119 isolate

AGGGGAATTGCGGCGGCTACACATGCAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGC
GGCGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGGATAACTACTGGAAACGGTAGCTAATACC
GCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATGAACCCAGGTGGGATTAGC
TAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAA
CTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAG
CCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGTAGCTAAT
AACTGCCAGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG
GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCC
CGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTA
GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCA
GGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAG
GCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTT
AAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAA
CCTTACCTGGCCTTGACATGTCTGGAATCCTGCAGAGATGCGGGAGTGCCTTCGGAATCAGACACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGAGATGTTGGGTTAAGTCCCGCACGAGCGCAACCCTGTCCTTGTTGCCGC
ACGTAATGGTGGGAACTCAGGGAGACTGCCGGGATAACCGAGAAAGGTGGGGATGACGGCCGGTCTTATGGG
CCTTCTGCCGGGCTACCACCGGGTTCAATGTGCCTACAAAGGTTCAGCTAGCGTTTGTAGGATCCCAAAAGGCG
CCCTATTCCGAGGGGAGTTGCATTCCCCCCGCCGCCGGAAACTTTTATTCAAAAAAAATTTTGTTAAAATTTCC
CCGGCTTTTACCCCCCCCCCCCCCGACAGGGGGTG

Figure 4: The sequence of the 16S rRNA gene of BT31 isolate

Serratia marcescens can be found in raw milk, water, soil; it is derived from the udders; it is an opportunistic pathogen of environmental origin and it is sometimes involved in mastitis. Management of herd health may be to control the contamination of *Serratia marcescens* in milk (Tormo et al., 2011). *Serratia marcescens* is an environmental bacterium capable of causing opportunistic infections in many animal species including mastitis in dairy cows. *Serratia* spp. can form biofilm on surfaces and produce heat resistant enzymes, thus they are capable of causing spoilage at different points of milk processing. *Aeromonas*

allosaccharophila was isolated from fish, water, animals and industrial sources (Stratev et al., 2012). *Aeromonas allosaccharophila* preferred nutrient-poor conditions for biofilm formation. Biofilm formation were affected by sources and species of bacteria, cell surface characteristics. Understanding the specific mechanisms by *Aeromonas* species may aid in preventing, treating disease outbreaks (Chenia et al., 2017).

Table 3: Homology sequence identity of bacterial isolates to strain in GenBank

Bacterial isolates	Nearest match BLAST search result	Query Cover (%)	Percentage Identity (%)	Accession number
BT17	*Pseudomonas oryzihabitants* strain 2.1	87	100	KY623077.1
	Pseudomonas sp. ZL03	90	98.90	KJ948663.1
	Pseudomonas aeruginosa strain Y33	90	98.89	KF641797.1
BT119	*Serratia marcescens* NPK2 2 20	98	98	MN691675.1
BT31	*Aeromonas veronii* strain PCG1	91	100	MN581681.1
	Aeromonas sorbia strain 109	91	100	MT730012.1
	Aeromonas caviae strain zj1	90	100	MF445125.1
	Aeromonas veronii strain JX16102	94	98.95	KY767538.1
	Aeromonas veronii strain BL16104	93	98.94	KY767536.1
	Aeromonas allosaccharophila strain 78-a blue	93	98.92	MN208205.1

3.3 Biochemical properties, extracellular enzymes producing capacities of the BT31 isolate

Results of the biochemical test were presented in Table 4. The BT31 isolate was Gram-negative; catalase and oxidase positive rods; citrate assimilation; urease negative bacteria; gelatin hydrolysis; fermentation of glucose and arabinose; no fermentation of lactose. A comparison between the findings of this study with those of Martinez-Murcia et al. (1992) shows that The features of biochemical characteristics are similar to that of the *Aeromonas allosaccharophila*. On the basis, allow to draw the conclusion the isolates belong to *Aeromonas allosaccharophila*. The results of the phenotypic characterization, based on morphological and biochemical tests, so it can be concluded that the BT31 isolate is actually as *Aeromonas allosaccharophila* BT31. Results in Figure 5 showed that *Aeromonas allosaccharophila* BT31 produced extracellular protease and lipase enzymes.

Table 4: Biochemical characteristics of the BT31 isolate and some strains of Aeromonas

Biochemical test	Bacterial strain				
	BM31	*A. caviae*[(1)]	*A. veronii*[(1)]	*A. sorbia*[(1)]	*A. allosaccharophila*[(2)]
Gram (-/+)	-	-	-	-	-
Catalase	+	+	+	+	+
Oxidase	+	ND	ND	ND	+
Gelatine	+	+	+	-	+
Citrate	-	+	+	+	-
Urease	-	+	-	-	-
Glucose	+	-	+	+	+
Lactose	-	+	+	+	-
Arabinose	+	+	+	+	-

ND: not detected "+": positive; "-": negative; [(1)]: Abbot et al. (2003); [(2)]: Martinez-Murcia et al. (1992)

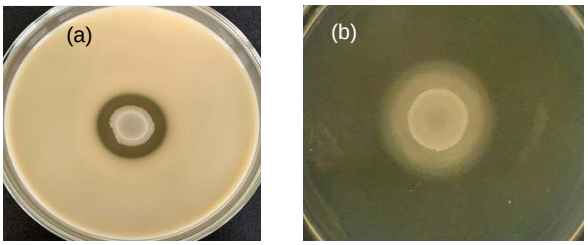

Figure 5: (a) Proteolytic activity of Aeromonas allosaccharophila BT31 in skim milk agar plate; (b) Lipolytic activity of Aeromonas allosaccharophila BT31 in Tween 80 hydrolysis agar plate

4. Conclusions

Based on the results, it can be concluded that the BT17, BT119 isolates had strong biofilm formation ability and were 100%, 98% of identity *Pseudomonas oryzihabitants* strain 2.1, *Serratia marcescens* NPK2 2 20 respectively. *Aeromonas allosaccharophila* BT31 was the strongest biofilm former and produced the extracellular proteolytic and lipolytic enzymes. Further experimental investigations are needed to the contamination of *Aeromonas allosaccharophila* BT31 in milk and find out facilitate improvements in the management of raw milk quality and dairy cattle health.

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