Iraqi Journal of Veterinary Sciences

www.vetmedmosul.com

Detection of protease and lipase activity in *Pseudomonas fluorescens* isolated from milk and surfaces as milk deteriorating indicators

A.H. Ahmed[1] and M.G. Hassan[2]

[1]Nineveh Health Directorate, [2]Department of Veterinary Public Health, College of Veterinary Medicine, University of Mosul, Mosul, Iraq

Article information

Article history:
Received 18 April, 2024
Accepted 10 July, 2024
Published online 19 September, 2024

Keywords:
Pseudomonas fluorescens
Exoenzymes
Milk

Correspondence:
M.G. Hassan
mghassan99@uomosul.edu.iq

Abstract

Raw milk exposure to many sources of contamination and cold storage is a big problem and may impact milk quality, especially the psychotropic *Pseudomonas fluorescens* a predominant bacterium causing milk spoilage. Some *P. fluorescens* strains can produce protease and lipase exoenzymes accelerating the milk spoilage process and reducing the shelf-life of milk and dairy products. Thirty-nine isolates of *P. fluorescens* isolated from raw cow milk, teat surfaces and milk tanks were examined to detect their abilities to produce both protease and lipase enzymes. These enzymes may increase the virulence of this bacterium based on the detection of both *aprX* (1434bp) and *lip* (1422bp) genes using polymerase chain reaction. Results of this study revealed the detection of the *aprX* gene in 36.8% of *P. fluorescens* isolates from raw milk and 81.8% in bacterium isolated from teat surfaces However, their ability to produce lipase enzymes was reduced by detecting *lip* genes in 26.3, 9.1, and 33.3% of *P. fluorescens* isolates from milk, teat surfaces and milk tanks respectively. These results confirm the potential of *P. fluorescens* to induce spoilage in milk and dairy products processed from milk through protease secretion which causes casein hydrolysis and indicates prognosis about the hygienic procedures during milking and processing affecting the milk shelf-life period.

DOI: 10.33899/ijvs.2024.148932.3621,

Introduction

P. fluorescens represents a saprophytic nonpathogenic with poor nutritional requirements and great diversity enabling them to survive in different environments including utensils used in the dairy production chain (1,2). These bacteria cause dairy product spoilage through their ability to produce some exoenzymes mainly proteases and lipases (3,4). Sometimes pseudomonas counts aren't indicative of the assessment of milk spoilage (5,6). Although some pseudomonas species become inactive after the thermal processing of milk like pasteurization their exoenzymes revealed high resistance to heat and affected milk shelf-life by casein and fat degradation (7,8). One of these enzymes is Alkaline zinc metalloprotease which consists of one zinc atom with eight atoms of calcium and a high molecular weight ranging between 21-25 kilodaltons their activity depends on the presence of calcium ions (9,10). AprX hydrolysez k-casein, para k-casein and beta-casein releasing plasmin and plasminogen which create unacceptable changes in milk (11). *P. fluorescens* produce lipolytic enzymes giving the rancid odor and flavor of dairy products (12,13). Lipases have a molecular mass between 30-50 KDa with optimum alkaline PH (14). The production of lipase is affected by the concentration and the type of nitrogen, iron and carbon whereas the presence of magnesium, zinc and iron decreases the activity of lipase (15,16). Molecular methods including polymerase chain reaction assay are used to monitor the impact of protease and lipase by detecting related genes represented by *aprX* and *lip* genes as spoilage indicators to evaluate milk quality (17). Local research studies the molecular features and antimicrobial sensitivity

of different bacterial isolates in some dairy products including some Pseudomonas spp. especially *P. aeruginosa* (18-20).

A few of them investigate the impact of *P. fluorescens* exoenzymes on milk quality therefore this study aims to screen the presence of *aprX* and *lip* genes in *P. fluorescens* isolated from cow milk teat surfaces and milk tanks in dairy farms in Nineveh province.

Materials and methods

Ethical approval

The research was conducted considering the ethical approval of the institutional Animal Care and Use Committee at the College of Veterinary Medicine, University of Mosul and included an authorized ID of UM.VET. 2023.102. Mosul, Iraq,2024.

Samples

The study included collecting one hundred fifty samples of cows' raw milk, teat surfaces swabs and milk tank swabs from Nineveh province dairy farms to detect the presence of *P. fluorescens* Thirty-nine positive isolates of *P. fluorescens* were distributed as 19,11, and 9 isolates from raw milk, teat surfaces and milk tanks respectively were examined to investigate their abilities to produce exoenzymes as spoilage indicators.

Isolation and identification

samples were cultivated on Cetrimide agar (Neogen/USA) incubated at 25°C for 24-48 hours then purified on Cetrimide agar and followed by biochemical tests (21). The *P. fluorescens* isolates were confirmed using a polymerase chain reaction depending on the*16srRNA* gene.

DNA extraction

P. fluorescens colonies were subjected to DNA extraction depending on the bacterial DNA preparation kit (AddBio, Korea) following the manufacturer's instruction protocol.

Polymerase chain reaction (PCR)

P. fluorescens protease and lipase activity were investigated using PCR assay depending on the detecting *aprX* and *lip* genes respectively. A specific primer was provided by (Macrogen/Korea). The primer consists of forward and reverse primers (22) with a molecular weight of 1434 bp and 1422 bp to *aprX* and *lip* gene respectively (Table 1). The thermal profile included an initial denaturation of 10 min. at 95ºC followed by 35 cycles of 95ºC for 45s, then annealing 58ºC, 55ºC for 45 sec. for *aprX* and *lip* gene respectively, extension at 72ºC for 1 min. and final extension of 72ºC for 5 min. with cooling at 4ºC. PCR products were analyzed by electrophoresis (1.5% agarose gel) (AddBio, Korea) with 3 µl GelRed dye (AddBio, Korea). 5 µl of each PCR product was loaded into the well of agarose gel. The electrophoresis was carried out at 75 volts for 1 hour. The band was identified using the Gel doc EZ image (Bio-Rad, USA).

Table 1: Oligonucleotide primers sequence of *P. fluorescens* exoenzymes genes used in the current study

Primers	Primer's sequence (5"-3")	Tmemperature (ºC)	Product size (bp)	Reference
aprX-F	TTATGTCAAAAGTAAAAGAC	58	1434	22
aprX-R	TCAGGCTACGATGTCACTG			
lip-F	ATGGGTRTSTTYGACTATAAAAACC	55	1422	22
lip-R	TTAACCGATCACAATCCCCTCC			

Results

Results showed the detection of protease and lipase exoenzymes genes of *P. fluorescens* strains isolated from cow raw milk, teat surfaces and milk tanks including *aprX* and *lip* genes. The *aprX* gene was detected in 58.9% (23/39) of isolates. The highest rate of *aprX* genes prevalence was found in the *P. fluorescens* isolates from teat surfaces (9/11) which was 81.8% compared to isolates from raw milk (7/19) which was 36.8%. The *lip* gene was detected in 23.1% (9/39) of isolates. *P. fluorescens* isolates from milk tanks revealed a higher percentage of the presence of *lip* gene (3/9) at 33.3% followed by isolates from raw milk (5/19) at 26.3% compared to *P. fluorescens* isolates from teat surfaces (1/11) 9.1% (Table 2 and Figure 1). The *aprX* and *lip* genes were detected at 1434 bp and 1422 bp respectively as shown in (Figures 2 and 3).

Table 2: Detections of protease and lipase activity in milk, teat surfaces and milk tanks

P. fluorescens sources	No.	Protease activity *aprX* gene		Lipase activity *lip* gene	
		No.	%	No.	%
Milk	19	7	36.8	5	26.3
Teat Surfaces	11	9	81.8	1	9.1
Milk tanks	9	7	77.7	3	33.3
Total	39	23	58.9	9	23.1

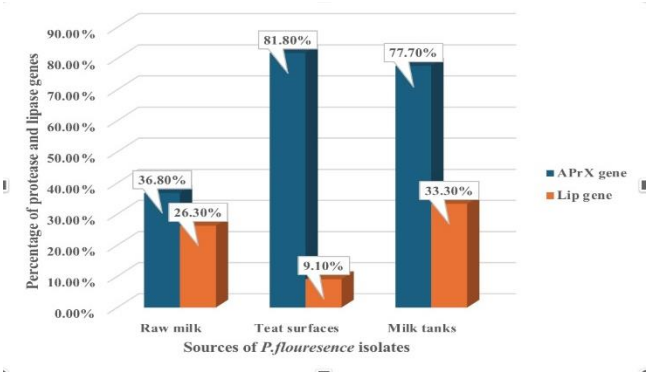

Figure 1: Percentage of protease and lipase genes in *P. fluorescens* isolates from milk, teat surfaces, and milk tanks.

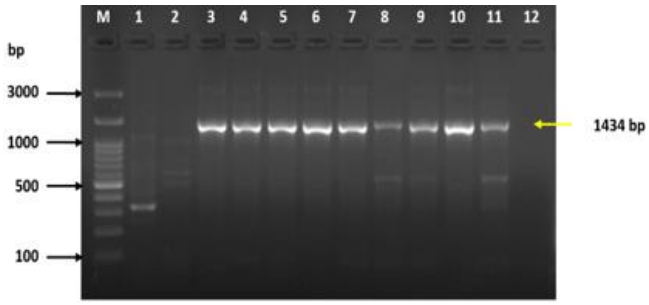

Figure 2: Amplified products of *aprX* gene of *P. fluorescens*, Lanes M represent 100 bp DNA marker, lane 1-2; negative samples, lane 3-11; positive samples at 1434 bp product size, lane 12; negative control.

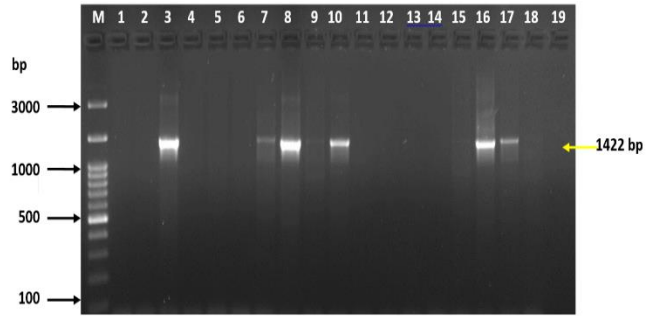

Figure 3: Amplified products of *lip* gene of *P. fluorescens*, Lanes M represent 100 bp DNA marker; lanes 1, 2, 4, 5, 6, 11, 12, 13, 14, 15, 18; negative samples, lanes 3, 7, 8, 9, 10, 16, 17; positive samples at 1422 bp product size lane 19; negative control.

Discussion

P. fluorescens is commonly isolated from soil, water and surfaces as a saprophytic bacterium (23,24) these species can secrete some enzymes including proteases and lipases exoenzymes and a number of these secreted enzymes depends on the *P. fluorescens* strain which highlighted the vast diversity of *P. fluorescens* isolates (25-27). *aprX* gene and *lip* gene were used as a marker to detect *P. fluorescens* protease and lipase activity in milk depending on PCR technique and distinguish proteolytic and lipolytic strains (28,29), to reduce the time for detecting *P. fluorescens* as deteriorating agents in raw milk and providing more flexible work to the dairy manager to assess milk quality (23,30). Therefore, PCR assay was used as a good and sensitive approach to detect the presence of protease and lipase enzymes as spoilage indicators to estimate the degradation of milk components including casein protein and lipids (31). The results of this study revealed the presence of the *aprX* gene in 36.8% of isolates from raw milk These results agree with the results obtained by De Longhi (32) who showed the presence of the *aprX* gene in 37% of *P. fluorescens* strains isolated from milk which indicates this bacterium's presence in farm animals' milking environments (33,34). *aprX* gene was observed in 71.2% of *P. fluorescens* isolates in bovine milk and the high presence of the *aprX* gene in cow raw milk is associated with sensory defects affecting the shelf life of milk (35). The presence of calcium is an essential factor for the potency and resistance of proteases (36,37). Also increased proteolytic activity in *P. fluorescens* may be related to the type of N-acyl homoserine lactone (AHL) either directly or indirectly (38). Although the *lip* gene was detected in raw milk and milk tank and teat surfaces their presence is at a lower limit than that of the *aprX* gene. These results disagree with Ribeiro (35) who revealed the lipolytic activity in 25% of *P. fluorescens* isolates from cow milk. In comparison the lipolytic activity of cow milk was evaluated in 9.3% of *P. fluorescens* isolates (39). It may be because protease was more resistant at a wide range of PH and temperatures (25,40,41) suggesting the adaptation of *P. fluorescens* strains to various environmental circumstances previous studies referred to the proteolytic activity of *P. fluorescens* in the late exponential and early stationary phase of growth (42-44). However, rapid and sensitive methods are essential to ensure product safety and perform risk assessment Preventive measures should be taken to reduce aprX biosynthesis in milk and dairy products.

Conclusion

Early detection of milk spoilage due to *P. fluorescens* is a significant predictive factor. This bacterium secretes exoenzymes represented by protease and lipase enzymes by identifying specific genes. Therefore, the activity of these exoenzymes is a limiting factor maintaining milk quality.

Acknowledgments

This research was supported using resources from the University of Mosul's College of Veterinary Medicine in Mosul, Iraq.

Conflict of interest

The authors confirm there was no conflict of interest.

References

1. Ganeshan G, Kumar MA. *Pseudomonas fluorescens*, a potential bacterial antagonist to control plant diseases. J Plant Interact. 2005;1(3):123-134. DOI: 10.1080/17429140600907043
2. Abdullah A, Esmael QA. Orientations of dairy producers and consumer attitudes (a field study in the province of Nineveh). Mesopotamia J Agric. 2018;46(4):109-120. DOI: 10.33899/magrj.2018.161538
3. Quigley L, O'Sullivan O, Stanton C, Beresford TP, Ross R, Fitzgerald GF, Cotter PD. The complex microbiota of raw milk. FEMS Microbiol Rev. 2013;37:664-698. DOI: 10.1111/1574-6976.12030
4. Chen L, Daniel RM, Coolbear T. Detection and impact of protease and lipase activities in milk and milk powders. Int Dairy J. 2003;13(4):255-275. DOI: 10.1016/S0958-6946(02)00171-1
5. Von Neubeck M, Baur C, Krewinkel M, Stoeckel M, Kranz B, Stressler T, Fischer L, Hinrichs J, Scherer S, Wenning M. Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. Int J Food Microbiol. 2015;211:57-65. DOI: 10.1016/j.ijfoodmicro.2015.07.001
6. Kumar H, Franzetti L, Kaushal A, Kumar D. *Pseudomonas fluorescens* a potential food spoiler and challenges and advances in its detection. Ann Microbiol. 2019;69:873-883. DOI: 10.1007/s13213-019-01501-7
7. Caldera L, Franzetti L, Van Coillie E, De Vos P, Stragier P, De Block J, Heyndrickx M. Identification, enzymatic spoilage characterization and proteolytic activity quantification of *Pseudomonas spp.* isolated from different foods. Food Microbiol. 2016;54:142-153. DOI: 10.1016/j.fm.2015.10.004
8. Marchand S, Vandriesche G, Coorevits A, Coudijzer K, De Jonghe V, Dewettinck K, De Vos P, Devreese B, Heyndrickx M, De Block J. Heterogeneity of heat-resistant proteases from milk Pseudomonas species. Int J Food Microbiol. 2009;133:68-77. DOI: 10.1016/j.ijfoodmicro.2009.04.027
9. Glück C, Rentschler E, Krewinkel M, Merz M, von Neubeck M, Wenning M, Scherer S, Stoeckel M, Hinrichs J, Stressler T, Fischer L. Thermostability of peptidases secreted by microorganisms associated with raw milk. Int Dairy J. 2016;56:186-197. DOI: 10.1016/j.idairyj.2016.01.025
10. Stuknytė M, Decimo M, Colzani M, Silvetti T, Brasca M, Cattaneo S, Aldini G, De Noni I. Extracellular thermostable proteolytic activity of the milk spoilage bacterium *Pseudomonas fluorescens* PS19 on bovine caseins. J Dairy Sci. 2016;99:4188-4195. DOI. 10.3168/jds.2016-10894
11. Deeth HC. Lipases from milk and other sources. In: Kelly AL, Larsen LB, editors. Agents of Change. Food Engineering Series. Switzerland: Springer; 2021. DOI: 10.1007/978-3-030-55482-8_10
12. Decimo M, Brasca M, Ordóñez JA, Cabeza MC. Fatty acids released from cream by psychrotrophs isolated from bovine raw milk. Int J Dairy Technol. 2017;70:339-344. DOI: 10.1111/1471-0307.12347
13. Rajmohan S, Dodd CE, Waites WM. Enzymes from isolates of *Pseudomonas fluorescens* involved in food spoilage. J Appl Microbiol. 2002;93(2):205-213. DOI: 10.1046/j.1365-2672.2002.0167
14. Capodifoglio E, Vidal AC, Lima JS, Bortoletto F, D'Abreu LF, Gonçalves AS, Vaz AN, Balieiro JC, Netto AS. Lipolytic and proteolytic activity of *Pseudomonas spp.* isolated during milking and storage of refrigerated raw milk. J Dairy Sci. 2016;99(7):5214-5223. DOI: 10.3168/jds.2015-10453
15. Woods RG, Burger M, Beven CA, Beacham IR. The aprX-lipA operon of *Pseudomonas fluorescens* B52: A molecular analysis of metalloprotease and lipase production. Microbiol. 2001;147(2):345-354. DOI: 10.1099/00221287-147-2-345
16. Morandi S, Pica V, Masotti F, Cattaneo S, Brasca M, De Noni I, Silvetti T. Proteolytic traits of psychrotrophic bacteria potentially causative of sterilized milk instability: Genotypic, phenotypic and peptidomic insight. Foods. 2021;10(5):934. DOI: 10.3390/foods10050934
17. Alkhafaje WK, Olama ZA, Ali SM. Molecular characterization and microbial resistance of different bacterial isolates in some dairy products. Iraqi J Vet Sci. 2022;36(2):333-339. DOI: 10.33899/ijvs.2021.130206.1764
18. Al-Rudhan AM, Khalil NK, Altaai NA. Evaluation of bacterial contaminants and heavy metals in cow and buffalo raw milk sold in Baghdad governorate. Iraqi J Vet Sci. 2021;35:101-105. DOI: 10.33899/ijvs.2021.131744.1999
19. Noomi B. Detection of virulence factors of *Pseudomonas aeruginosa* in different animals by using bacteriological and molecular methods. Iraqi J Vet Sci. 2019;32(2):205-210. DOI: 10.33899/ijvs.2019.153851
20. Scarpellini M, Franzetti L, Galli A. Development of PCR assay to identify *Pseudomonas fluorescens* and its biotype. FEMS Microbiol Lett. 2004;236(2):257-260. DOI: 10.1016/j.femsle.2004.05.043
21. Roberts D, Greenwood M. Practical food microbiology. 3rd ed. UK: Blackwell Publishing Ltd; 2008. 273-274 pp.
22. Martins ML, Pinto UM, Riedel K, Vanetti MC. Milk-deteriorating exoenzymes from *Pseudomonas fluorescens* 041 isolated from refrigerated raw milk. Braz J Microbiol. 2015;46(1):207-217. DOI: 10.1590/S1517-838246120130859
23. Vidal AC, Netto AS, Vaz AN, Capodifoglio E, Gonçalves AS, Rossi GM, Ruiz VA. *Pseudomonas spp.*: Contamination sources in bulk tanks of dairy farms. Pesquisa Vet Bras. 2017;37(9):941-948. DOI: 10.1590/s0100-736x2017000900008
24. Bellassi P, Rocchetti G, Morelli L, Senizza B, Lucini L, Cappa F. A milk foodomics investigation into the effect of *Pseudomonas fluorescens* growth under cold chain conditions. Foods. 2021;10(6):1173. DOI: 10.3390/foods10061173
25. Anbu P. Characterization of an extracellular lipase by *Pseudomonas koreensis* BK-l07 isolated from soil. Prep Biochem Biotechnol. 2014;44:266-280. DOI: 10.1080/10826068.2013.812564
26. Baglinière F, Matéos A, Tanguy G, Jardin J, Briard-Bion V, Rousseau F, Robert B, Beaucher E, Gaillard JL, Amiel C, Humbert G, Dary A, Gaucheron F. Proteolysis of ultra-high temperature-treated casein micelles by AprX enzyme from *Pseudomonas fluorescens* F induces their destabilization. Int Dairy J. 2013;31(2):55-61. DOI: 10.1016/j.idairyj.2013.02.011
27. Meshaan R, Alhaji T. Effect of ohmic heating treatment on different properties of whole cow milk. Mesopotamia J Agric. 2022;50(2):68-76. DOI: 10.33899/magrj.2022.133744.1171
28. Machado SG, Bazzolli DS, Vanetti MD. Development of a PCR method for detecting proteolytic psychrotrophic bacteria in raw milk. Int Dairy J. 2013;29(1):8-14. DOI: 10.1016/j.idairyj.2012.09.007
29. Martins ML, de Araújo EF, Mantovani HC, Moraes CA, Vanetti MC. Detection of the apr gene in proteolytic psychrotrophic bacteria isolated from refrigerated raw milk. Int J Food Microbiol. 2005;102(2):203-211. DOI: 10.1016/j.ijfoodmicro.2004.12.016
30. Decherni S, Benjelloun H, Lebeault JM. Effect of modified atmospheres on the growth and extracellular enzyme activities of psychrotrophs in raw milk. Eng Life Sci. 2005;5(4):350-356. DOI: 10.1002/elsc.200520082
31. Rosenau F, Jaeger K. Bacterial lipases from Pseudomonas: Regulation of gene expression and mechanisms of secretion. Biochim. 2000;82(11):1023-32. DOI: 10.1016/s0300-9084(00)01182-2
32. De Longhi R, Bruzaroski SR, Eleodoro JI, Poli-Frederico RC, Fagnani R, Ludovico A, de Santana EH. Presence of aprX gene in *Pseudomonas spp.* from refrigerated raw milk and their proteolytic ability. Semin Cienc Agrar. 2020;41(4):1421-1426. DOI: 10.5433/1679-0359.2020v41n4p1421
33. Decimo M, Morandi S, Silvetti T, Brasca M. Characterization of Gram negative psychrotrophic bacteria isolated from Italian bulk tank milk. J Food Sci. 2014;79:2081-90. DOI: 10.1111/1750-3841.12645
34. De Oliveira GB, Favarin L, Luchese RH, McIntosh D. Psychrotrophic bacteria in milk: How much do we really know?. Braz J Microbiol. 2015;46(2):313-321. DOI: 10.1590/S1517-838246220130963
35. Ribeiro Júnior JC, de Oliveira AM, Silva FG, Tamanini R, de Oliveira AM, Beloti V. The main spoilage-related psychrotrophic bacteria in

الكشف عن فعالية البروتيز واللايبيز في جراثيم الزوائف المتألقة المعزولة من الحليب الخام والأسطح كمؤشرات على فساد الحليب

أحمد حمدي أحمد[١] و منتهى غازي حسن[٢]

[١]دائرة صحة نينوى، [٢]فرع الصحة العامة البيطرية، كلية الطب البيطري، جامعة الموصل، الموصل، العراق

الخلاصة

يتعرض الحليب إلى العديد من الملوثات خلال مراحل تصنيع منتجاته وتحت ظروف الخزن بدرجة حرارة التبريد تتأثر نوعية الحليب وبدرجة كبيرة بتواجد جراثيم الزوائف وخاصة الزوائف المتألقة كونها الجراثيم السائدة والمسببة للفساد. تمتلك بعض عترات الزوائف المتألقة القابلية على إنتاج الإنزيمات الخارجية المحللة للبروتين والمحللة للدهون والتي تعجل من عملية فساد الحليب وتقلل مدة حفظ الحليب. أخذت تسع وثلاثون عزلة من جراثيم الزوائف المتألقة المعزولة من حليب الأبقار الخام وأسطح حلمات الضرع وخزانات الحليب للكشف عن قابليتها على إنتاج إنزيم البروتيز واللايبيز اعتمادا على وجود الجينات *aprX* و *lip* بتقنية تفاعل البلمرة المتسلسل وإعطاء حزم بوزن جزيئي ١٤٢٢ و ١٤٣٤ زوجا قاعديا لكل منهما بالتتابع .أظهرت النتائج وجود جين *aprX* في ٣٦,٨%من عزلات الزوائف المتألقة المعزولة من الحليب الخام و ٨١,٨% من عزلات الزوائف المأخوذة من أسطح حلمات الضرع بينما كانت قابلية هذه العزلات على إنتاج إنزيم اللايبيز منخفضة من خلال وجود جين *lip* في ٣٦,٣، ٩,١ و ٣٣,٣% من عزلات الزوائف المتألقة المعزولة من الحليب الخام وأسطح حلمات الضرع وخزانات الحليب على التوالي ،وتؤكّد هذه النتائج قدرة هذه العزلات على أحداث الفساد في الحليب الخام بتأثير إنزيم البروتيز من خلال تحلل بروتينات الكازئين والتي ممكن اعتمادها كدليل للتنبؤ بحدوث فساد الحليب وتقييم الشروط الصحية أثناء عمليات الحلب وتصنيع منتجات الحليب وفترة حفظ الحليب.

refrigerated raw milk. J Dairy Sci. 2018;101(1):75-83. DOI: 10.3168/jds.2017-13069

36. Zhang C, Bijl E, Svensson B, Hettinga K. The extracellular protease aprX from Pseudomonas and its spoilage potential for UHT Milk: A review. Compr Rev Food Sci. 2019;18:834-852. DOI: 10.1111/1541-4337.12452
37. Ertan H, Cassel C, Verma A, Poljak A, Charlton T, Aldrich-Wright J, Omar S, Siddiqui K, Cavicchioli R. A new broad specificity alkaline metalloprotease from a *Pseudomonas sp.* isolated from refrigerated milk: Role of calcium in improving enzyme productivity. J Mol Catal B Enzym. 2015;113:1-8. DOI: 10.1016/j.molcatb.2014.12.010
38. Liu M, Wang H, Griffiths MW. Regulation of alkaline metalloprotease promoter by N-acyl homoserine lactone quorum sensing in *Pseudomonas fluorescens*. J Appl Microbiol. 2007;103:2174-2184. DOI: 10.1111/j.1365-2672.2007.03488
39. Baur C, Krewinkel M, Kranz B, von Neubeck M, Wenning M, Scherer S, Stoeckel M, Hinrichs J, Stressler T, Fischer L. Quantification of the proteolytic and lipolytic activity of microorganisms isolated from raw milk. Int Dairy J. 2015;49:23-29. DOI: 10.1016/j.idairyj.2015.04.005
40. Boran R, Ugur A. Partial Purification and characterization of the organic solvent-tolerant lipase produced by *Pseudomonas fluorescens* RB02-3 isolated from milk. Prep Biochem Biotech. 2010;40:229-241. DOI: 10.1080/10826068.2010.488929
41. Paludetti LF, Kelly AL, Gleeson D. Effect of thermoresistant protease of *Pseudomonas fluorescens* on rennet coagulation properties and proteolysis of milk. J Dairy Sci. 2020;103:4043-4055. DOI: 10.3168/jds.2019-17771
42. Dufour D, Nicodème M, Perrin C, Driou A, Brusseaux E, Humbert G, Gaillard JL, Dary A. Molecular typing of industrial strains of *Pseudomonas spp*. isolated from milk and genetic and biochemical characterization of an extracellular protease produced by one of them. Int J Food Microbiol. 2008;125:188-196. DOI: 10.1016/j.ijfoodmicro.2008.04.004
43. Abdallah KS, Hasan GM. Evaluation the microbial method used to judge the quality control of pasteurized milk. Mesopotamia J Agric. 2018;46(4):327-332. DOI: 10.33899/magrj.2018.161555
44. Nicodème M, Grill JP, Humbert G, Gaillard JL. Extracellular protease activity of different Pseudomonas strains: Dependence of proteolytic activity on culture conditions. J Appl Microbiol. 2005;99(3):641-648. DOI: 10.1111/j.1365-2672.2005.02634