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Protease-Producing Pseudomonas in Cold-Stored Milk: Mechanisms of Quality Deterioration: A Review

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Abstract

This review article addresses a major problem in the dairy industry, namely the role that protease-producing bacteria, Pseudomonas, play in the deterioration of the quality of cold-stored milk. Although cooling slows the growth of most bacteria, it creates an ideal environment for the proliferation of psychrotrophic bacteria, such as Pseudomonas. These bacteria are widespread in dairy environments and can contaminate milk after pasteurization. The main problem is the ability of Pseudomonas to produce heat-resistant protease (proteolytic) and lipase (lipid-lyzing) enzymes, which remain active even after pasteurization. These enzymes target and break down essential milk proteins (such as casein), resulting in: Changes in texture: gelation, increased viscosity and undesirable texture, changes in flavor: release of bitter peptides due to casein degradation, free fatty acids that cause rancid or soapy flavors due to lipolysis, synergistic effects: Proteolysis and lipoprotein work together to accelerate the degradation process and produce unwanted flavor compounds at a faster pace. The article reviews in detail the protease systems in Pseudomonas, which include mineral enzymes (e.g. AprX) and serine enzymes, and how their production is regulated at the genetic and environmental level (e.g., temperature, pH, quorum sensing). It also discusses the impact of storage conditions, especially temperature fluctuations and oxygen availability, on bacterial growth and enzymatic activity.

In terms of detection and monitoring, the article points out that traditional methods (microbial transplantation) are slow and impractical for routine monitoring. Therefore, biochemical methods (e.g., enzymatic activity assays) and molecular methods (e.g., PCR and metagenomics techniques) are being used for rapid and accurate diagnosis of degrading potential.

Keywords: Microbial spoilage, Pseudomonas, Proteases, Proteolysis, Cryophilic enzymes, Casein.

1. Introduction

The cold storage of raw milk at refrigeration temperatures, commonly adopted in the dairy industry, has proven to be an effective measure to slow down microbial growth and extend shelf life (1). However, it can inadvertently favour the development of spoilage microorganisms. Principal among them, Pseudomonas species, are widely distributed in dairy environments. Pseudomonas spp. remained the dominant flora in pasteurized milk stored in large milk tanks at a temperature of 3 °C, both in total counts and in percentage of positive detected tubes (2). These organisms are adept at growing and producing spoilage enzymes such as proteases and lipases at low temperatures (3). They have shown adaptability to dairy environments and

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spoilage capabilities similar to those of Pseudomonas spp. widely distributed in various niches (4). Given extreme adaptability to different environmental conditions and milk spoilage traits with high economic impact, attention is focused on improving the detection and control of protease-producing Pseudomonas spp. associated with raw milk at post-pasteurization stages, where they often gain access and can cause severe dairy product quality defects (5).

Pseudomonas spp. produce a broad range of extracellular proteolytic enzymes that target milk caseins and exacerbate the economic impact of proteolytic spoilage potential (6). The spoilage activity on milk proteins leads to the formation of bitter-tasting peptides and destabilisation of emulsions in dairy products. Moreover, Pseudomonas spp. Enzymes are produced independently of growth and are thermostable. Consequently, even post-pasteurization contamination of milk with Pseudomonas spp. poses a considerable quality risk (7,8).

2. Background on Pseudomonas in Dairy Environments

Nowadays, microorganisms are often associated with the degradation of food quality, causing significant economic and financial losses (9). A prime example of this effect is the dairy industry, where the fast spoilage of refrigerated milk limits its shelf life and storage period (10). Freezing and refrigeration at sub-zero to low temperatures have been widely recognized as convenient methods of extending food shelf life. However, refrigeration of raw dairy products has a unique disadvantage. During cold storage, protease-producing Pseudomonas species can contaminate and spoil raw milk with the production of milk-clotting enzymes, leading to unwanted chemical changes (5,11).

3. Protease Systems in Pseudomonas

Pseudomonas spp. are well-known protease producers that secrete either endo- or exoproteases capable of degrading caseins and whey proteins (12). The specificity of milk-Borne Pseudomonas proteases has been extensively studied. Commonly identified proteases include thermolysin-related zinc metalloproteases (e.g., pseudomonas leucine (PleA) and XaaPro) and serine proteases (e.g., pseudomonas alkaline (AlpA) and elastase (ElaB)) (13). These enzymes exhibit distinct substrate specificities, targeting various regions of the caseins: PleA hydrolyzes the three-casein protein, αS1-casein, following the cleavage of the −Phe−Asp− bond in the amino acid sequence, which can result in milk protein gelation and the release of bitterness-associated peptides 2. Upon considering these findings, it should be possible to establish a relationship between protein proteolysis, soya milk gelation, and the release of off-flavor compounds in milk (14).

The proteases produced by Pseudomonas spp. are secreted through different pathways and are expressed upon exposure to various environmental stimuli (15). The predominant proteases secreted into purified soya milk are metalloproteases regulated by the quorum-sensing signal (16). The specific environmental cues influencing protease gene expression (including temperature and pH) and the involvement of several regulatory networks in the control of Pseudomonas protease synthesis have already been identified (15). Overall, protease synthesis is controlled at transcriptional and post-transcriptional levels by regulatory factors that modulate mRNA stability and/or translation (17).

3.1. Endoproteases and Exoproteases

Proteins are secreted by Pseudomonas spp. generally via classical pathways. Exoprotease production generally occurs outside the cell; however, some strains also produce intracellular serine proteases that hydrolyze caseins. Regulations and expression of protease genes in Pseudomonas spp. isolated from cold-stored milk are determined and compared to other genera of bacteria (18,19).

In Gram-negative bacteria, three types of export routes are generally identified: the signal peptide-dependent general secretion system (Sec) or twin-arginine translocase system (Tat), and an independent type system (T1SS/T2SS/T3SS/T4SS) (20). Secretion of large proteins occurs through T1SS, while T2SS is known to expel hydrolytic enzymes such as proteases, lipases, and amylases 3. In Pseudomonas spp. isolated from milk, the T2SS is the unique pathway involved in the secretion of endo-type and exo-type proteases (21).

3.2. Regulation of Protease Expression

Proteases are the most important deteriorating agents produced by psychrotrophic Pseudomonas in cold-stored milk. Environmental conditions regulate their production, which researchers have modeled to facilitate milk quality prediction during distribution (22). In milk, the proteases secreted by Pseudomonas cause significant casein degradation, resulting in premature spoiling linked to bitter peptide formation and undesirable texture. It is believed that such activity correlates with gross visual changes in product quality (3).

The proteolytic activity in milk by psychrotrophic bacteria has been extensively evaluated. Not only has the proteolytic activity of psychrotrophic strains in pasteurized milk been assessed by inoculating milk with each strain and then incubating it at 4 °C for five days, but the strains known to affect the physical stability of cold-stored milk have been studied as well (23). According to these evaluations, P. fluorescens and H. alvei are the fastest growing psychrotrophic strains in milk at low temperatures (12). The activity of AprX and Ser1, both well-known thermostable proteases, was confirmed for five out of seven strains, and other notable findings included weaker proteolytic activity by P. poae and C. freundii, and stronger activity by P. fluorescens, C. joostei, and S. marcescens. P. fulva and H. alvei, by contrast, do not produce thermostable proteases at all (24). Regarding Pseudomonas strains, extracellular proteases commonly accumulate in the supernatants of stationary phase cultures and, in particular, heat-stable protease production shows a clear temperature dependence. One strain of P. fluorescens, for example, secretes lower levels of protease at 4 °C than at higher temperatures, which might explain the absence of gelation in milk stored at that temperature (25).

4. Mechanisms of Milk Quality Deterioration

Pseudomonas proteolytic activity is frequently implicated in alterations to milk quality characteristics. Cold-storage of milk allows for bacterial growth due to the nutrient-rich environment provided, leading to proteolytic spoilage. Early studies outlined the main hydrolytic enzymes produced by Pseudomonas in milk—metallo- and serine-type proteases, which can act on all four caseins (αs1, αs2, β, κ) and are stable in milk at 4 °C (4,8). These enzymes produce small peptides from medium-length casein fragments that reduce the viscosity of milk gels. Simultaneously, the generation of bitterness precursor peptides occurs. Hydrolysis events targeting αs1- and β-casein are paramount due to the higher surface exposure of these proteins. Changes in flavor and odour due to the release of free fatty acids from membrane lipids by lipolytic enzymes have also been reported (26). Pseudomonas proteolytic and lipolytic systems actively cooperate, with residual peptides generated by proteolysis further stimulating and accelerating lipolysis. The consequent formation of long-chain fatty acids increases the rate of off-flavour development, while also enhancing rancid odour attributes. While lipolysis can proceed independently, combined proteolysis/lipolysis interactions generate undesirable characteristics sooner than individual substance action (11).

4.1. Casein Hydrolysis and Pommeled Texture

In milk, Pseudomonas proteases preferentially target the αS1-casein and β-casein fractions, leading to the release of polypeptides and free amino acids from caseins (3). Casein hydrolysis disrupts the protein framework, impairing gelation, increasing viscosity,

and negatively affecting mouthfeel. Subsequent syneresis and phase separation result in a pommeled texture, which is considered a defect. Proteolysis also liberates bitterness precursors, contributing to undesirable organoleptic changes (27,28).

4.2. Lipolysis and Off-Flavors

Milk spoils when it develops off-flavors, which may occur during cold storage of pasteurized milk. The main cause of off-flavors in dairy products is lipolytic activities by psychrotrophic microorganisms such as Pseudomonas spp., frequently found in raw milk and dairy-processing environments (2). Pseudomonads are capable of growth at low temperatures and can produce a wide array of extracellular enzymes, including lipases, compatible with the conditions prevailing in stored milk (12). During lipolysis, triglycerides are hydrolyzed into free fatty acids and glycerol. Free fatty acids having low odor thresholds are responsible for rancid, soapy, or savory off-flavors. The combined activities of proteases and lipases from Pseudomonas spp. enhance the development of off-flavors in milk on the cold chain (29).

4.3. Synergistic Effects of Proteolysis and Lipolysis

Proteolysis and lipolysis exert major detrimental effects on cold-stored milk quality. Proteolysis of caseins by Pseudomonas proteases alters textural properties, while lipolysis produces off-flavours through the release of free fatty acids. These two processes do not arise independently. Proteolysis substantially impacts flavour alongside texture since it influences the degradation of flavour precursors. In parallel, lipolytic free fatty acids interact with peptide fragments and other metabolites generated by proteolysis, producing a wide array of aromatic compounds. The resulting quality loss accumulates through cascading effects between texture, flavour compounds, and spoilage progression (3,12).

5. Influence of Cold Storage Parameters

5.1. Temperature Fluctuations and Growth Kaps

Temperature excursions during cold storage of milk, such as those resulting from the opening of tankers at processing plants, breaks in the cold chain before retail distribution, or thawing and refreezing, can permit psychrotrophic bacteria to grow. The subsequent contamination of batch milk with Pseudomonas spp. and other proteolytic microorganisms poses a significant risk of quality deterioration during cold storage (30).

Thermal events that impose a temperature above the maximum growth temperature of mesophilic bacteria yet remain well below the optimum of Pseudomonas can be especially conducive to the growth of milk-borne psychrotrophs with a psychrotrophic Pseudomonas population. Experiments with temperature profiles commonly encountered post-processing and during distribution of chilled milk suggested that time–temperature integrators linked to protease activity could be a useful tool for predicting quality deterioration (3).

5.2. Packaging and Oxygen Availability

Pseudomonas spp. are aerobic microorganisms, and their presence in cold-stored milk is significantly influenced by packaging and oxygen availability. Oxygen exposure during filling, the composition of the headspace gas above the liquid, and ambient atmospheric conditions during storage alter the microbial community, fostering not only Pseudomonas spp. but also other potential spoilage microorganisms. Different materials and technologies for packaging, as well as modified atmospheres, can alter the oxygen content in packaged products, thus influencing Pseudomonas growth and associated spoilage (18,31).

Pseudomonas may be cultivated under aerobic and microaerophilic conditions. Fresh industrial milk packaged in non-vented containers (such as gable-

top cartons made of paper-polymer composite), and filtered through a 0.2 µm membrane, is able to form microaerophilic headspaces (≈ 5–11% O2; ≈ 5–12% CO2), resulting in microbial community shifts. During the first week, coliforms grow and become the prevailing group (marginally detectable), but by 14 days, Pseudomonas prevails without detection of coliforms, a pattern that holds in-situ Monitoring O2 for longer periods also reveals Pseudomonas (3,14).

6. Detection and Monitoring of Protease Activity

Detection and monitoring of protease activity are crucial in understanding spoilage processes in milk and dairy products. Psychrotrophic bacteria, such as Pseudomonas spp., produce extracellular proteases that degrade casein, leading to reduced cheese yields and tainting (12). These bacteria often form biofilms composed of mixed species that enhance their persistence and spoilage potential. Biofilm formation involves adherence, proliferation, and dispersion stages, with extracellular matrix modulation (32). Quorum-sensing molecules like N-acyl-homoserine lactones regulate protease production in Gram-negative bacteria, especially at high cell densities during late exponential growth. Monitoring protease activity is important for maintaining milk quality, as biofilms can protect bacteria from cleaning procedures and facilitate ongoing enzyme production, contributing to spoilage despite refrigeration (3).

Conventional microbiological approaches to protease detection rely on colony-forming unit counts (CFU) on selective and differential media, coupled with protease screening assays. These methods can provide an indirect estimate of the proteolytic potential of microbial communities in chilled milk. CFU counts offer time-to-detection ranging from 2–7 days, depending on the spoilage organism and medium. Accordingly, such approaches are impractical for routine quality control (33). Quantitative biochemical assays are available that employ milk casein, sodium dodecyl sulfate, and buffer/substrate mixtures with a colorimetric endpoint. Casein- and peptide-based zymography are also employed (34). Major protease genes have been characterized and linked to the proteolytic phenotype of psychrotrophic isolates. Gene and transcriptomic markers have been utilized to track spoilage during storage, while qPCR and metagenomic techniques can monitor the functional potential of specific communities. Omics-based approaches have been applied to the study of whole microbial communities, although functional linkages remain uncertain (35).

6.1. Conventional Microbiological Methods

Proteolytic enzyme production by Pseudomonas species in cold-stored milk is evaluated using conventional microbiological methods. Microbial populations are enumerated by determining colony-forming units (CFU) on selective or differential count media (12). Such media can promote the growth of specific contaminants as well as signal their potential spoilage mechanisms and influence storage and distribution parameters. Pseudomonas spp. Proteolytic activity is indicated by the formation of clear zones around colonies grown on milk plates or the use of gelatin as a solidifier (3). Although selective culture-based detection methods are slow, requiring several days for time-to-detection, they remain simple, practical, and widely utilized for routine quality monitoring (36).

6.2. Biochemical and Molecular Approaches

Proteolytic enzymes are of major concern for the dairy industry because of their role in the Spoilage of extended shelf-life milk. Colony-forming unit counts using selective/differential media followed by protease screening assays are conventional microbiological methods for detecting proteolytic P. fluorescens in milk (12). However, they can be time-consuming and may not allow prompt enforcement of corrective measures at the collection or processing stages. Biochemical or molecular approaches for detecting protease-producing

Pseudomonas spp. in cold-stored milk are valuable supplements to standard enumeration methods (37).

Protease activity can be detected in milk using colorimetric or fluorimetric assays for peptides released from casein, gelatin, or azocasein (3). Zymography using casein or gelatin gels identifies the type or activity of proteases produced, and specificity tests for other substrates further characterize enzyme profiles. The presence of selected genetic or transcriptomic markers linked to protease-coding regions might indicate potential proteolytic activity (37). Quantitative polymerase chain reaction (qPCR) offers a useful approach for assessing spoilage-associated bacterial populations. Sequences of 16S rRNA, 23S rRNA, and specific genes are widely used for monitoring and tracing Pseudomonas spp. in dairy products, along with metagenomic approaches. When combined with targeted metabarcoding or other omics-based methodologies, such techniques are suited for investigating functional potential and metabolic traits related to spoilage (38).

7. Control Strategies and Mitigation

Cold-stored milk can be protected against proteolytic Pseudomonas spp.—which are spoilage threats in the supply chain—by a multifaceted approach targeting initial contamination, enzyme activity control, and shelf-life prediction. (6)

Improving hygiene practices and equipment design in dairy processing plants can limit the introduction and spread of Pseudomonas spp. (12). Cleaning-in-place (CIP) protocols should include sporadic validation of cleaning efficiency and of cleansing agents' compatibility with milk-contact surfaces. Antimicrobials that do not alter milk's taste can reduce Pseudomonas counts without severely affecting milk quality (2). The safety of potential biopreservation solutions should adhere to current safety regulations and the relevant recommendations of the International Dairy Federation (IDF). Approaches include the use of competitive micro-organisms such as bacteriocin-producing lactic acid bacteria or phages attacking specific spoilage bacteria (39).

Shelf-life prediction models center on protease activity, storage temperature profiles, and pack type. Models designed to prevent proteolytic and lipolytic spoilage of various products in different chilling scenarios are also available. Risk assessment (RA) frameworks provide supportive models based on shelf-life estimates and other data. Decision-support tools tailored to the dairy sector would facilitate wider uptake of the approach (40).

7.1. Hygiene and Processing Interventions

7.2. Antimicrobial Strategies and Biopreservation

Antimicrobial hurdles to Pseudomonas could be directed toward prolonging the shelf life of cold-stored milk. SQF-certified antilisterial and antibiofilm approaches could serve to constrain Pseudomonas growth; however, no bisphenol-based preservatives are currently authorized for direct addition to raw or pasteurized liquid milk (6). Biopreservation using GRAS micro-organisms is yet another avenue for safe intervention; the available datum, albeit limited and tempered with practical constraints, indicates that it bears exploration with a view to a regulated commercial application. Such exploration should encompass the assessment of potential risks to sensory quality, whether by the introduction of off-flavours or the preclusion of desirable flavour development (2).

In addition to hygiene protocols and antimicrobial interventions, mathematical models predictive of shelf life based on temperature history and proteolytic activity may support industry operations. By combining these models with hazard analysis, risk assessment frameworks could furnish processors with the capacity to appraise and mitigate spoilage risk on shipment arrival (3).

7.3. Shelf-Life Prediction and Risk Assessment

Protease activity models have been incorporated into mathematical models to evaluate shelf-life under various storage temperature profiles, thus enabling the prediction of storage time before the end-of-shelf-life limit is reached. Various predictive models link changes in protease activity to overall quality (3). Risk assessment strategies based on microbial spoilage have been designed and successfully implemented in several European countries for different food matrices, integrating models used for estimating the safety of processors. The design and delivery of this guidance and tools by governments and academic institutions is considered key to industry reach (2).

8. Conclusion

Protease-producing Pseudomonas spp. can survive pasteurization and proliferate in milk stored under refrigeration. Their enzymes are implicated in quality defects observed in cold-stored milk—particularly bitterness and abnormal texture—and in the destabilization of ultra-high-temperature-sterilized milk. Contamination of milk and dairy products occurs from multiple sources. In addition to raw milk and ingredients such as cream, starter cultures, and carbon dioxide, Pseudomonas may be introduced via water, air, lubricants, and contaminated equipment. Equipment cleaning and sanitation must therefore be effective in removing biofilms and planktonic cells from the farm to the filling line.

Deterioration of milk quality during extended cold storage involves the production of proteolytic and lipolytic enzymes by psychrotolerant bacteria. Protease-producing Pseudomonas and Bacillus, common in raw and pasteurized milk, are major contributors. The milk industry has a considerable interest in the spoilage potential of Pseudomonas spp. Since various Pseudomonas species and strains differ, monitoring broader proteolytic activity can provide a less complex overview. Consequently, the section examines factors influencing proteases produced by Pseudomonas spp. occurring in milk and their effect on quality.

Cold-storage temperature fluctuations, allowing growth before re-establishing low temperature, promote proteolytic activity. Minimum growth temperature for vegetative Pseudomonas is usually 4 °C; incubation at higher temperatures can enable protease production after chilling. Temperature excursions suppress the population of non-thermoresistant microorganisms. Under specific conditions, Pseudomonas spp. can sustain growth at 0 °C. Various countries are considering an upper storage limit of 4 °C; however, milk is routinely stored at higher temperatures, prolonging exposure to ATP-degrading psychrotrophic bacteria. Temperature profiles during transport and warehousing also influence proteolytic characteristics under cold storage.

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