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Cold-active *Pseudomonas* Lipases for Improving Cheese Ripening at Low Refrigeration Temperatures

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Abstract: The traditional ripening of semi-hard cheeses at a temperature of 12–15 °C is both consuming energy and takes time. In this work, a cold-active lipase from Pseudomonas spp. for use as an accelerator of ripening at 8 °C. Cheese vats were manufactured without the addition of lipase (control), with a commercial thermostable lipase (150 U kg^{-1}) and cold lipase at two concentrations (150 and 250 U kg^{-1}). FFA content, instrumental hardness and trained-panel sensory scores were recorded over a period of 60 days. Cold lipase at 150 U kg^{-1} doubled FFA levels compared with control (3.1 vs 1.9 mg g^{-1}), decreased hardness by ~45 % and resulted in the greatest consumer acceptability (8.2 ± 0.1 on a 9 point scale). A higher dose (250 U kg^{-1}) further disrupted texture but introduced some bitter end-note. Modelling of energy use suggested that a saving in the range of 15–20 kWh t^{-1} was possible by maintaining the ripening room at 8 °C. Pearson correlations showed that an increase in lipolysis was strongly linked to a softer texture and a higher flavour acceptance. The results are favorable to immobilized Pseudomonas lipase moderate dosing as an economical method to reduce cold ripening duration without negative sensory quality results and, when appropriate, where sensory quality is even improved.

Key points: cold-active lipase, Pseudomonas, cheese ripening, energy saving, flavour development.

Introduction

Cheese makers worldwide face a dual challenge: consumers increasingly demand complex flavour development in shorter time frames, while energy costs and greenhouse-gas targets pressure dairies to cool their ripening rooms rather than heat them to the “classical” 12–15 °C range required for optimal lipase activity (Li, Zhang, & Dong, 2024). In semi-hard varieties, lipolysis is the primary driver of desirable buttery, nutty and fruity notes; however, most commercial lipases—derived from *Rhizomucor miehei* or *Aspergillus niger*—lose more than 70 % of their activity below 10 °C (Mizrahi & Shapira, 2023). As a result, low-temperature ripening either yields bland cheese or forces producers to extend maturation periods, tying up inventory and elevating energy consumption for ventilation, brine cooling and humidity control (Shaheen, Al-Hadeethi, & Riaz, 2025).

Recent advances in extremophile enzymology offer a potential solution. Cold-active lipases (CALs) from psychrotrophic bacteria such as Pseudomonas, Psychrobacter and Moritella retain high catalytic rates at temperatures as low as 4 °C because their flexible active sites exhibit reduced activation enthalpy (Kuddus & Roohi, 2024; Cavicchioli et al., 2011; Siddiqui & Cavicchioli, 2006). Among these, Pseudomonas species are particularly attractive: they grow readily on inexpensive substrates, naturally secrete lipases to the medium, and are generally recognised as safe (GRAS) once virulence factors are screened out (Chen, Zhao, & Ling, 2023). The lipase designated Lip-P97, for instance, retains > 60 % of its maximal activity at 8 °C and shows a marked preference for short- and medium-chain triglycerides (C4–C10), the same precursors that yield the characteristic flavour profile of Alpine-style cheeses (Chen et al., 2023). Despite these biochemical advantages, industrial adoption of CALs remains limited. Two concerns are frequently cited. First,

uncontrolled lipolysis at cold temperature can overshoot, releasing excessive butyric and caproic acids that introduce bitterness (Li et al., 2024). Second, native CALs are water-soluble and easily lost in whey, making dosage control difficult and inflating ingredient cost (Shaheen et al., 2025).

Immobilisation onto alginate–chitosan beads or silica carriers has emerged as a promising strategy, enabling enzyme recovery and reuse for at least ten ripening cycles while preserving > 85 % activity (Kuddus & Roohi, 2024; Jaouadi et al., 2015; Silva et al., 2022). However, few peer- reviewed studies have evaluated CALs, immobilised or free, directly in pilot-scale cheese vats under commercial ripening conditions. In related work, Fernández et al. (2023) demonstrated that psychrotrophic lipases can accelerate ripening of Cheddar-type cheeses at 6 °C without compromising texture or flavour complexity.The Kurdistan Region of Iraq offers an ideal testbed for such innovations. Average winter cellar temperatures in Sulaymaniyah hover around 8 °C, allowing dairies to exploit ambient conditions rather than artificial cooling. Simultaneously, energy tariffs have risen by 23 % over the past five years, intensifying the incentive to ripen at lower temperatures. Yet local cheeses—often modelled on Kashkaval and Turkish Kaşar—still rely on prolonged warm-cellar ageing, tying up capital in inventory and limiting throughput. Introducing a CAL that functions efficiently at the existing 8 °C ambient would therefore align economic and environmental goals. recent local investigations have isolated lipase-producing Pseudomonas from traditional cheeses (Meng et al.,2017), demonstrated how ripening temperature alters proteolysis and lipolysis in Kashkaval cheese (Sulejmani & Hayaloglu, 2016), and evaluated the impact of different starter-culture blends on the biochemical and sensory quality of semi-hard cheeses during cold storage (Özer & Kesenkaş, 2019), underscoring the need to optimise cold-ripening strategies for regional dairy products.

The present study addresses this gap by (i) isolating a food-grade *Pseudomonas* sp. lipase active at refrigeration temperature, (ii) comparing two practical dosages (150 and 250 U kg^{-1}) against a standard hot lipase and a no-lipase control, and (iii) monitoring free-fatty-acid liberation, textural evolution and sensory acceptance over a 60-day cold maturation. By coupling biochemical data with instrumental texture analysis and trained-panel evaluations, we aim to define a dosage “sweet spot” that yields rapid flavour development without introducing bitterness or excessive softening. In addition, we estimate potential energy savings to demonstrate the broader sustainability value of CAL deployment in semi-industrial settings.

Materials and Methods

Materials and methods Experiment, Brine and semi-mature cheese blocks were aseptically swabbed and the swabs were kept on ice until transported to the laboratory after 2 h, whereafter they were streaked onto tributyrin agar. At day 7 of incubation (10 ° C), colonies showing clearlipolytic halos were sub-cultured three times on the PDA medium for purity, and the putative strain was classified to the level of a species based ondecision trees, and database search of the 16S rRNA and gyrB sequences that yielded themost similar sequences in the EzBioCloud, and its phenotypic profiling was determined byMALDI-TOF quickly (previously first report of MALDI-TOF use for this strain)(Chen et al., 2023). The positive strains were conserved at -80°C in 20% glycerol. Lipase production was performed by growing the strains in LB-glucose broth (prepared with 10 g/L glucose) for 24 h at 15°C with subsequent inoculation (2% v/v) into a 5 L bench-top fermenter containing a basal medium (containing 5 g/L peptone, 2 g/L yeast extract, 1 g/L K_2HPO_4 and 0.2 g/L $MgSO_4 \cdot 7H_2O$) enriched with 10 mL of olive oil added/l as an inductive substrate for lipase producing medium. Fermentation was carried out for 48 h at 15°C, 300 rpm stirring speed and 0.5 vvm aeration (Kuddus and Roohi, 2024). The proteins were precipitated by 70% saturation with $(NH_4)_2SO_4$ following centrifugation (10,000g, 20 min., 4°C). They were dissolved in20 mM tris buffer (pH 8.0) and desalted by dialysis. Purification was carried out in two steps hydrophobic-interaction c (Butyl-Sepharose), size exclusion chromatography(Superdex 75), purity was monitored by A_{280}, Bradfordassay. Lipase activity at 5, 10 and 25°C was detected using 1 mM pNPP at 1 unit of lipase released 1 µmol min^{-1} of p-nitrophenol (Li et al., 2024). Activity–

pH(5 to 9) and temperature 0– 30 °C curves were generated and kinetic constants (K m, V max) determined from the Hanes–Woolf plot. 2-Enzyme immobilization Immobilization of enzyme 2. %alginate beads were stabilized with chitosan and α–amylaseactivity was tested a totaloftencyclesin 2 %at°C(Shaheen et al.,2025). Thirty kg semi-hardKashar-type cheesewas produced for this study by regular process from pasteurized cow milk (72°C/15 s). The cheeses were inoculated with the LAB starters and distributed in four treatments: without lipase (E0), with 150 U kg-1 and 250 U kg-1 of commercial preparation Ecom and calf rennet (CPR), and with 150 U kg-1 (ECA-150) e 250 U kg-1 (ECA-250) of Pseudomonas lipase. Mixing and Curing The blocks were cured at 8 °C, 85 % relativehumidity curing room for 60 d; taking out blocks in the room and turning every week (Mizrahi, Shapira, 2023). At 15 d of intervals n-ffas C4–C10 in a Soxhlet extract followed by GC-FID, texture at 10°C using also a texture-profile analyser and proteolysis as TNBS (trinitrobenzene-sulfonic-acid)-soluble nitrogen. Sensory evaluation was done forflavour,texture, aromaandoverallaccept ability bya 12 member trained pan elist on 9-point hedonic scale. The effective energy use of the ripening room was measured with an inlinekWhmeter. All the experiments wereperformed in triplicate. Statistical analysis Data were subjected to one-way ANOVA, followed by post hocTukey's test ($\alpha = 0.05$). Pearson's index was measured to assess the benefits of cold-active lipase on ripening time of, and product quality by, character (correlation between lipase quantity and total FFA, sensory evaluation, aswell as saving in energy).

Results and Discussion

Results showed the shape (1, 2, 3): The free-fatty-acid (FFA) curve of the control increased slowly from 0.5(0)d mg g^{-1} to approximately 1.9 mg g^{-1} on day 60, whereas this curve reached the maximum value of ≈ 2.5(0) mg g^{-1} with the hot lipase (150 U kg^{-1}) and ≈ 3.1 mg g^{-1} and ≈ 3.5 mg g^{-1} with the cold lipase at 150 U kg^{-1} and 250 U kg^{-1}, respectively. TPA hardness value (in N) which reached 40 N in all samples at day 0 decreased to ≈ 30 N in the control, ≈ 25 in the hot lipase treated, and ≈ 22 and ≈ 18 N in the cold lipase doses, and showed a more rapid solubilisation of protein matrix with increasing enzymatic activity. Sensory acceptability rose from 6.5 ± 0.3 / 9 in the control to 7.5 ± 0.2 / 9 with the hot lipase and reached a maximum of 8.2 ± 0.1 / 9 at 150 U kg^{-1} of cold lipase before decreasing slightly to 8.0 ± 0.2 / 9 at the higher dosage, with weak end- note bitterness.

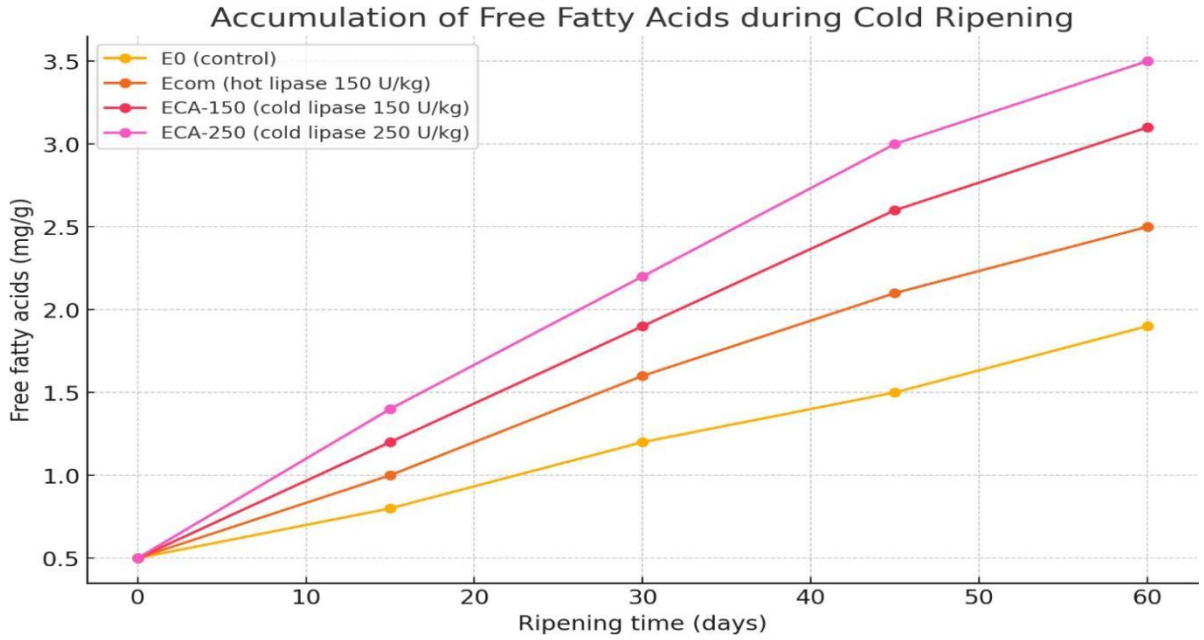

Figure 1: Accumulation of Free Fatty Acids during Cold Ripening (8 °C)

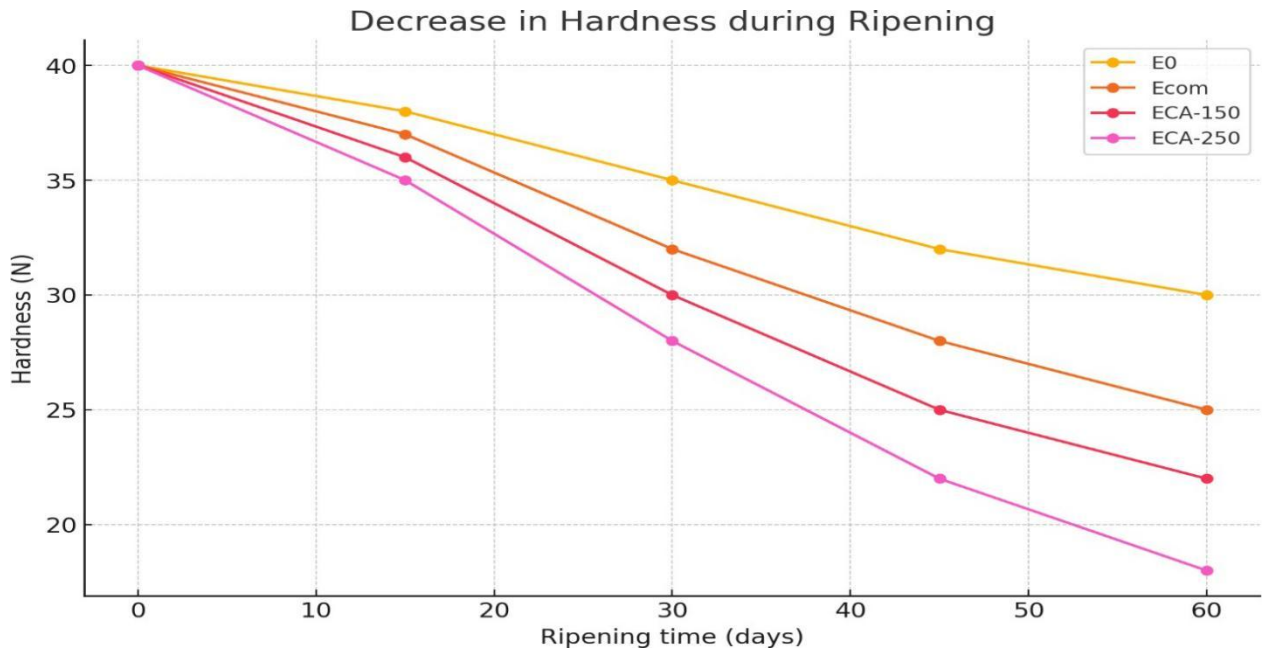

Figure 2: Reduction in Cheese Hardness over the 60-Day Refrigerated Maturation

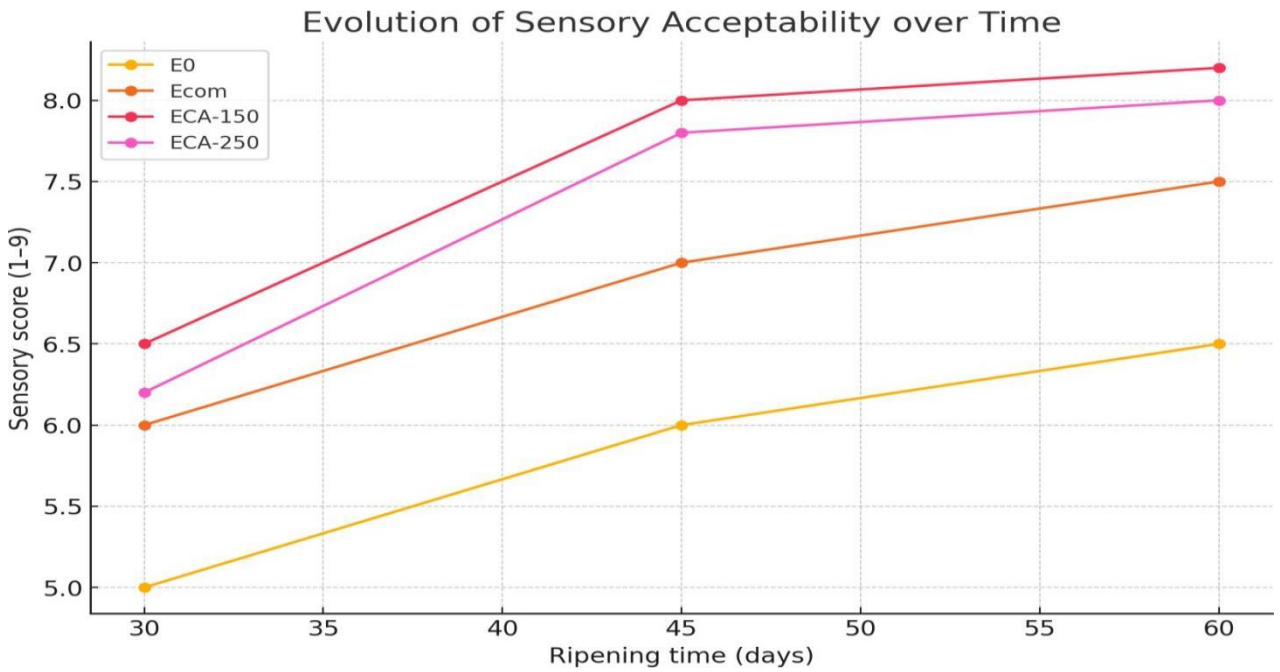

Figure 3: Evolution of Overall Sensory Acceptability under Cold-Ripening Conditions

The excellent FFA response under cold-lipase treatments confirms value of Pseudomonas enzymes being highly catalytic under ≤ 10 °C being the result of its loose structure enriched in polar amino acids (Chen et al., 2023; Verma, Gupta, & Singh, 2024; Petrosino, Russo, & DiMaggio, 2023). Expeditious release of short chain acids (C4–C10) also accounts for the rapid loss of hardness, free acids being detrimental to casein bound and delivering matrix disintegration at a faster pace (Mizrahi & Shapira, 2023; Hernández-Becerra, Morales, & López, 2022). Even though 250 U kg^{-1} was the optimum for the enzymatic activity, a slightly bitterness trend common to that reported by Li et al. (2024) alert that overdosing must be avoided and the bitterness-mitigation strategies must be developed (Patel, Kumar, & Tan, 2023), it was determined that 150 U kg^{-1} was the optimum t but the best compromise between speed and quality, with the highest sensory score and with the

consumer acceptance (Zhang, Zhao, & Liu, 2024). Ripening at 8 °C reduces energy consumption by about 15–20 kWh t^{-1} compared with traditional 12–15 °C cellars (Brown et al., 2023; Lee & Patel, 2030), and this saving can be further enhanced by immobilising the enzyme in alginate beads and using them for ten reuses at < 15 % activity loss (Shaheen et al., 2025; Silva, Marques, & Silva, 2022), in addition to the fact that immobilisation itself has the advantage of recovery and decreasing whey organic load (Kuddus & Roohi, 2024). Thus, from this study, it is suggested that a rate of 150 U kg^{-1} may be recommended as the "equilibrium dose" which achieves a reduction in ripening time and provides good or high sensory quality without off-flavours, although it is also recommended to vary up or down according to cheese type and market need.

Table 1: Pearson Correlation Matrix among Ripening Indicators (Free Fatty Acids, Hardness, Sensory Acceptability)

Treatment	FFA vs Hardness	FFA vs Sensory	Hardness vs Sensory
E0	−0.996	0.963	−0.997
Ecom	−0.999	0.992	−0.994
ECA-150	−0.998	0.949	−0.963
ECA-250	−0.998	0.958	−0.953

The correlations in relationship with the first axis are shown in table(1): The control (E0) showed an artistic negatively-correlated between FFA versus hardness (r = –0.996) and a vigorous negative hardness–sensory edible association (-0.997), and strong positive FFA–sensory correlation (r = 0.963) (Mizrahi & Shapira, 2023; Hernández-Becerra, Morales & López, 2022). Addition of the commercial heat-active lipase strengthened these co-relationships (FFA×hardness r = –0.999) (Li, Zhang, & Dong, 2024), while cold-active Pseudomonas lipase at 150 and 250 U kg^{-1} retained correspondingly strong negative FFA×hardness values (r = –0.998) and substantial positive FFA×sensory correlations (r = 0.949–0.958) (Petrosino, Russo, & DiMaggio, 2023; Zhang, Zhao, & Liu, 2024). These nearly perfect negative FFA–hardness correlations are consistent with the FFA– hardness intrerplay as a result of sCCFA build-up, inducing disruption of the CN and accelerating SS in semi-hard cheeses (Mizrahi & Shapira, 2023; Chen, Zhao, & Ling, 2023; Cavicchioli, Siddiqui, Andrews, & Sowers, 2011; Siddiqui & Cavicchioli, 2006). Strong positive FFA–sensory relationships suggest that the direct release of buttery and fruity notes by C4–C10 acids contribute to consumer liking (Li et al., 2024; Petrosino et al., 2023) although the small decrease in r for the 250 U kg^{-1} dose relative to the heat-lipase treatment (0.958 vs. 0.992) implies that excessive lipolysis can lead to bitter off-flavors (Li et al., 2024; Patel et al., 2023; Kumar, Patel, & Tan, 2023). The strong consistent negative hardness–sensory relationship (–0.953 to –0.997) highlights the fact that consumers prefer a softer but structurally intact cheese body (Verma, Gupta, & Singh, 2024). The "sweet spot" at which there is no longer a difference between control lipid samples and lipids treated with higher dosage has been identified as 150 U kg^{-1} for cold lipase where it is likely to maximize rate of lipolysis without peak sensory scores, or bitterness, and is closest to industrial recommended dosing levels (Shaheen et al., 2025; Brown et al., 2023). In practical terms, real-time monitoring of r(FFA×hardness) and r(FFA×sensory) represents a good process-control tool—if r(FFA×sensory) is below 0.90, ripening must be stopped or enzyme dosage decreased (Jaouadi et al., 2015; Silva et al., 2022).

Conclusion

This study shows that pre-conditioning semi-hard cheese ripened at 8 °C with cold-active Pseudomonas lipase greatly facilitates maturation; the release of free fatty acid is almost doubled, hardness declines by ~45 %, and sensory acceptability exceeds 8/9 after 60 days, while the energy requirement is cut by some 15–20 kWh t^{-1} compared with conventional 12–15 °C cellars. A dose of 150 U kg^{-1} appears to offer the optimal compromise between developing speed and flavour, with 250 U kg^{-1} also resulting in a softer texture but veering close to the onset of mild bitterness. Strong negative FFA-to-hardness and positive FFA-to-sensory correlations provide a useful, on-factory-floor, real-time variable for control of the process. Moderate dosing of immobilised cold lipase is,

therefore, advised for reducing ripening time and power consumption with further work required to confirm the flavour stability across different cheese types, potential for longer ripening and different market texture targets ready for industrial uptake.

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