SPANDIDOS PUBLICATIONS
WORLD ACADEMY OF SCIENCES JOURNAL 7: 111, 2025

Biochemical and functional characterization of a *Penicillium purpurescens* milk-clotting enzyme as an animal rennet alternative

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Received June 16, 2025; Accepted September 5, 2025

DOI: 10.3892/wasj.2025.399

Abstract. Fungal milk-clotting enzymes (MCEs) provide a sustainable and efficient alternative to animal-derived rennet in cheese production, providing advantages, such as scalability and compatibility with diverse dietary needs. The present study aimed to optimize the production and characterize the enzymatic properties of an extracellular MCE from *Penicillium purpurescens*. The highest enzyme yield was achieved using solid-state fermentation with wheat bran as the substrate under conditions of pH 6.0, 60˚C, a 1% inoculum size and supplementation with 1% $CaCl_2$ on the 5th day of cultivation. The enzyme was purified 17.2-fold with a 20% recovery rate through acetone precipitation and ion-exchange chromatography, and SDS-PAGE analysis revealed a molecular mass of 29 kDa. Kinetic analyses determined a K_m value of 5×10^{-2} M, indicating high substrate affinity. The purified enzyme exhibited optimal activity at 45-55˚C and a broad pH range (pH 5-6), along with notable stability under varying conditions. The analysis of metal ion effects revealed the enhancement of activity by Ca^{2+} and Mg^{2+}, while it revealed the inhibition of Hg^{2+} and Cu^{2+}. These properties, combined with a high specificity for κ-casein, highlight the potential of the enzyme for industrial dairy applications, particularly in processes requiring robust and adaptable clotting agents. On the whole, the present study demonstrates that *Penicillium purpurescens* may be a highly promising fungal strain for MCE production, demonstrating superior clotting efficiency compared to other screened strains.

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Key words: fungal milk-clotting enzyme, *Penicillium purpurescens*, cheese production

Introduction

Cheese production has long relied on animal-derived rennet, a complex of proteolytic enzymes primarily chymosin extracted from the stomach lining of young ruminants. However, growing global demand for cheese, coupled with ethical, religious, dietary and environmental concerns, has accelerated the search for sustainable alternatives to animal rennet. Vegetarianism, the rise of vegan products and issues regarding animal welfare have intensified the need for microbial milk-clotting enzymes (MCEs), which provide a scalable and animal-free solution for coagulating milk during cheese manufacture (1). Among microbial sources, filamentous fungi have attracted increasing attention due to their high enzyme productivity, the ease of cultivation under diverse conditions, and the ability to secrete extracellular enzymes with milk-clotting potential (2,3).

The genus *Penicillium*, well-known for its enzymatic versatility, includes several species with the capacity to produce proteases that exhibit milk-clotting activity. However, a number of microbial proteases are often accompanied by high levels of non-specific proteolytic activity, which can adversely affect cheese texture and flavor due to excessive casein hydrolysis (4). Therefore, an ideal milk-clotting enzyme should possess a high milk-clotting activity (MCA)-to-proteolytic activity (PA) ratio, ensuring specificity toward κ-casein the key substrate responsible for casein micelle destabilization and curd formation. In this context, exploring novel fungal strains capable of producing enzymes with a favorable MCA/PA profile is a critical step toward developing viable alternatives to animal rennet (5).

Recent advances made in enzyme biotechnology have enabled the identification, production and biochemical characterization of fungal MCEs with industrial potential. Solid-state fermentation (SSF), in particular, has emerged as an efficient technique for fungal enzyme production due to its cost effectiveness, high product yield, and alignment with sustainable waste management practices. Agricultural residues such as wheat bran and rice husks serve as inexpensive substrates for fungal growth, rendering SSF a preferred method for enzyme production in resource-limited settings (6). The optimization of SSF parameters, including pH, temperature, inoculum size, metal ion supplementation and fermentation time is essential to maximize enzyme yields and ensure functional integrity (7).

In the present study, an extracellular MCE produced by *Penicillium purpurescens* was optimized under SSF conditions using wheat bran as a substrate. The effects of processing variables, such as incubation temperature, medium pH, calcium ion supplementation and inoculum size were systematically evaluated to identify the optimal conditions for MCE production. The enzyme was subsequently purified through acetone precipitation and ion-exchange chromatography, and its molecular mass was estimated via SDS-PAGE. Kinetic characterization, including the determination of the Michaelis-Menten constant (*Km*), provided insight into substrate affinity and enzymatic efficiency, which are crucial for evaluating its industrial applicability.

The enzyme exhibited a molecular mass of 29 kDa, high specificity toward κ-casein, and optimal activity in the temperature range of 45-55˚C across a broad pH spectrum (5.0-10.0), which are desirable traits for industrial cheese-making applications. Furthermore, the enzyme demonstrated notable thermal and pH stability, rendering it suitable for various dairy processing conditions. The influence of metal ions on enzyme activity revealed significant a enhancement in of Ca^{2+} and Mg^{2+}, which are commonly present in milk and known to stabilize casein micelles, while heavy metals, such as Hg^{2+} and Cu^{2+} were inhibited; these findings are findings consistent with those of previous research on microbial MCEs (8,9).

Compared to conventional calf rennet, the enzyme derived from *Penicillium purpurescens* demonstrates comparable clotting performance with a lower proteolytic degradation of milk proteins, indicating its potential as a functional rennet substitute. The food industry is increasingly moving towards greener, animal-free and sustainable processes, and fungal MCEs provide a promising platform for innovation in this domain. Moreover, fungal enzymes have been deemed to be generally recognized as safe (GRAS) by regulatory bodies such as the FDA, further supporting their adoption in dairy processing (FAO/WHO, 2021) (10).

It was thus hypothesized that *Penicillium purpurescens* can produce a MCE with a favorable MCA/PA ratio under optimized solid-state fermentation conditions. The present study aimed not only to characterize the biochemical and functional properties of a novel MCE from *Penicillium purpurescens*, but also to lay the groundwork for its application in commercial cheese production. Although the genus *Penicillium* includes several species known for their proteolytic and clotting activities, there are limited or no detailed reports on the milk-clotting potential of *P. purpurescens*, rendering the present study a novel contribution to the search for effective microbial coagulants. However, further is required to focus on recombinant expression systems, enzyme immobilization techniques and pilot-scale cheese trials to establish its efficacy across diverse cheese types and processing conditions.

Materials and methods

Microorganisms and culture conditions. All the fungal strains used in the present study were obtained from the laboratory of the Department Microbial Chemistry at the National Research Centre in Cairo, Egypt. The strains were maintained by sub-culturing them on slants of solid Czapek Dox medium containing: glucose, 30 g/l; NaNO, 2 g/l; KH_2PO_4, 1 g/l; $MgSO_4 \cdot 7H_2O$, 0.5 g/l; KCl, 0.5 g/l and agar, 20 g/l (BDH Chemicals Limited) at 28˚C and then stored at 4˚C. The inoculum size was adjusted by manually counting the spores.

Screening of different organisms for MCE production. All organisms were cultivated in liquid Czapek Dox medium (BDH Chemical, Ltd.), containing (g/l): glucose, 30; NaNO, 2; KH_2PO_4, 1; $MgSO_4$.7 H_2O, 0.5; KCl, 0.5 and following incubation at 28˚C for 5 days, the cultures were centrifuged at 5,000 x g, for 10 min at room temperature to obtain cell-free supernatants as crude enzyme extracts. These extracts were assayed for milk-clotting activity (MCA) by mixing 0.5 ml enzyme with 4.5 ml pre-warmed (35˚C) reconstituted skim milk containing $CaCl_2$, and the clotting time was recorded visually. The activity was expressed in arbitrary units relative to the time required for visible coagulation. To assess non-specific proteolysis, proteolytic activity (PA) was determined by incubating enzyme with azocasein, (Merck KGaA) (or casein), followed by trichloroacetic acid (TCA; El-Gomhouria Company), precipitation and measurement of soluble peptides spectrophotometrically. The ratio of MCA to PA was then calculated for each organism to evaluate enzyme suitability for cheesemaking. Organisms with high MCA and high MCA/PA ratio were considered the best candidates and selected for further purification and characterization, as shown Table I.

Chemicals and reagents. All chemicals and analytical-grade reagents used in the present study were procured from Merck KGaA. Skimmed milk powder (for MCA assays) was obtained from a local commercial supplier (El-Nasr Co. for Dairy Products). Wheat bran substrate was provided by ARMA Food Industries.

Optimization of fermentation conditions

Evaluation of different culture media for MCE production. The effects of various culture media on MCE production were investigated using both submerged fermentation (SmF) and SSF under static and shaking conditions. A total of 10 liquid media were evaluated, including natural potato broth, Sabouraud dextrose broth, Czapek Dox broth, modified Czapek Dox with 5% sucrose and peptone, modified Czapek Dox with 5% sucrose and $NaNO_3$, starch nitrate broth, malt extract broth, yeast extract peptone dextrose (YPD) broth, synthetic potato dextrose agar (PDA) broth and a specific medium (Merck KGaA) (11). For each medium, 50 ml were inoculated with 1 ml spore suspension (7×10^6 spores/ml) and incubated at 28˚C for 7 days under static and shaking (180 rpm) conditions. Additionally, eight agro-industrial residues, namely wheat bran, sugarcane bagasse, banana peel, guava seeds, corn stover, pomegranate peel, tangerine peel and sawdust (purchased from the local Arma Food Industry, Egyptian Company for solid waste recycling) were screened as solid substrates for MCE production in SSF. For SSF experiments, 5 g of each substrate were moistened with 5 ml distilled water, sterilized (121˚C for 15 min) and then inoculated with 1 ml of the same spore suspension. The mixtures were homogenized and incubated at 28˚C for 7 days. For enzyme

Table I. Screening of different organisms for MCE production.

Tested strains	Milk-clotting activity MCA/ml (mean ± SD)
Scopulariopsis brevicaulis - natural potato	-
Penicillium javanicum - exo- dox	-
Aspergillus flavus - dox	-
Aspergillus flavus - natural potato	-
Penicillium purpurescens	190±3.4
Penicillium oxalicum - exo	-
Penicillium oxalicum - endo	-
Aspergillus ustus - dox	-
Aspergillus ustus - potato	-
Aspergillus phoenix - exo	-
Aspergillus phoenix - endo	-
Erytherma sp. - exo- dox	-
Penicillium oxalicum - endo	-
Curvularia sp. DHE5	60.8±2.1
Aspergillus Fumigatus	-
Aspergillus tamari DHE10 homogenate	-
Streptomyces aurecens	-
Penicillium politans	-
Aspergillus oryzae static - dox	-
Aspergillus oryzae static - PDA	-
Aspergillus oryzae shaking - dox	-
Aspergillus oryzae shaking - PDA	102±3.4
Noh2	45±1.2
New *Aspergillus sp.* - endo	-

Values are expressed as the mean ± SD of enzyme activity as triplicates. Exo, exocellular enzyme; Endo, endocellular enzyme; Exo-dox, exocellular enzyme in Czapek Dox medium; Dox, Czapek Dox medium; PDA, potato dextrose agar medium; Noh2 and DHE10, codes for unidentified fungal isolates used in the present study.

extraction, the content of each flask was mixed with 100 ml of 0.05 M extraction buffer (Na-citrate, citric acid, pH 6.0) (1:10, w/v). The culture was mechanically agitated at 180 rpm in a shaking incubator (Benchmark Scientific Inc.) at 28˚C for 30 min. The mixture was then filtered to separate the mycelia from the medium. The supernatant (crude-enzyme extract) developed was utilized for MCE activity and determination of protein content, the method described by Nema *et al* (12). Through this optimization process, the ideal cultivation conditions for *Penicillium purpurescens* were determined to be as follows: A moisture content maintained between 60-70%, an aeration level of 75%, shaking at 180 rpm, a temperature range of 28-30˚C, an initial pH of 6.0-6.5 and an incubation period of 5 days.

Initial pH. The effect of incubation pH on MCE production was examined in the pH range of 3.0 to 10. The media pH was adjusted using either dilute HCl or dilute NaOH (El-Gomhouria Company) prior to autoclaving the bran (13).

Incubation temperature. Temperature was optimized for the growth and production of MCE by incubating the flasks containing the inoculated bran with pH 6, at 20, 25, 30, 35, 40 and 45˚C for 7 days (13).

Fermentation time. The MCE activity was monitored at 24-h intervals for up to 240 h. MCE production at optimum pH 6 and temperature 28˚C was followed. At the end of each time point, the extent of MCE production was determined as described by Vishwanatha *et al* (13).

Inoculum size. A total of 5 g of wheat bran was moistened with 5 ml distilled water (pH 6) and autoclaved. The substrate was inoculated with different volumes of the same spore suspension (0.25, 0.5, 1, 2, 3, 4 and 5 ml). The flasks were incubated at 25˚C for 5 days.

Analysis of the effect of the $CaCl_2$ concentration on MCE. Different volumes of the substrate (milk + $CaCl_2$) were tested (0.125, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5 and 3 ml) against the same volume of the enzyme.

Determination of MCA. The MCA was determined using the method previously described by Kumar *et al* (14), based on the appearance of the first discontinuous particles and expressed in Soxhlet units (SU). The enzyme solution was incubated at 35˚C for 10 min before being added to skim milk (10% wt/vol, containing 10 mM $CaCl_2$ (Merck KGaA), which had been pre-incubated at the same temperature for 5 min. The reaction was terminated once discontinuous particles formed. The MCA was calculated using the following formula: SU=(2,400 x VS x N)/(T x VE), where VS represents the skim milk volume (ml), N is the MCE dilution factor, T is the milk-clotting time (seconds) and VE is the volume of the MCE (ml) used in the assay.

Determination of PA. PA was determined using *N,N*-dimethyl casein (DMC) (Merck KGaA) as the substrate, following the method described in the study by Fan *et al* (15). In brief, 45 μl enzyme solution were combined with 45 μl substrate solution (10 mg/ml DMC in 20 mM potassium phosphate buffer, pH 5.8) and incubated at 35˚C for 30 min. The reaction was terminated by the addition of 350 μl of 100 mg/ml trichloroacetic acid (TCA) (El-Gomhouria Company), followed by incubation on ice for 20 min to precipitate undigested proteins. The samples were centrifuged at 2,000 x g for 15 min at 22˚C, and the absorbance of the supernatant was measured at 280 nm (UNICO advanced UV-VISIBLE spectrophotometer). A blank was prepared for each sample by adding TCA before the enzyme to correct for non-enzymatic hydrolysis. A total of one unit of PA [measured in proteolytic units (PU)] was defined as the amount of enzyme that releases 1 μg tyrosine per minute at 35˚C, using a tyrosine extinction coefficient (ε) of ml/μg for quantification:

$$\text{PU (U/ml)} = \frac{A280\ \text{nm}}{0.005} \times 1.44\,\frac{1}{30} \times \frac{1000}{45}$$

MCE purification. The crude enzyme solution was fractioned by acetone and the active fraction with high MCA was further purified by passing through a column (1.5x40 cm) of DEAE-cellulose (Pharmacia Fine Chemicals AB) pre-equilibrated with 0.02 M sodium phosphate buffer at pH 6. The elution of protein was then carried out by the batch-wise addition of 40 ml portions of increasing molarities (0.0-0.4 M) of

NaCl in 0.02 M phosphate buffer at pH 6. Fractions of 5 ml each were collected at room temperature (25˚C) at a flow rate of ~20 ml/h. The eluted fractions from the DEAE-cellulose column were dialyzed against cold distilled water to remove excess NaCl. The resulting purified enzyme solution was aliquoted into multiple test tubes, each containing 2 ml, and stored at -20˚C for later use. Under these storage conditions, the enzyme remained stable for >1 month. However, subjecting the enzyme to more than three freeze-thaw cycles resulted in approximately a 21% loss of activity. The enzyme remains active in the refrigerator for up to 1 week (16).

Determination of MCE purity and concentration. The molecular weight of the enzyme was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the method described by Laemmli (17). A 12% acrylamide gel was prepared, and protein bands were visualized by staining with Coomassie Blue R-250 (Merck KGaA) at room temperature for 1 h, followed by de-staining until clear background was obtained. Zymography was performed in two steps: First, native-PAGE was conducted using a 12% polyacrylamide gel without SDS, as described by Snoek-van Beurden and Von den Hoff (18) with minor modifications. Samples were loaded without prior boiling.

Effect of pH and temperature on Penicillium purpurescens MCE. To evaluate the effect of pH on the MCA of *Penicillium purpurescens*, the enzyme activity was assayed at different pH values ranging from 3.0 to 8.0. Substrate buffer solutions were prepared using: 0.05 M citrate buffer for pH 3.0-6.0, and 0.05 M phosphate buffer for pH 6.5-8.0. Each reaction mixture adjusted to the desired pH. The optimal pH was defined as the pH exhibiting the highest MCA. To assess the influence of temperature on MCA, enzyme assays were performed at temperatures ranging from 20 to 80˚C.

Effect of metal ions on Penicillium purpurescens MCE. The *Penicillium purpurescens* MCE was dissolved in solutions containing 10 or 50 μM Na^+, Ca^{2+}, Mg^{2+}, Hg^{2+} and Cu^{2+}, respectively, and incubated for 30 min at 40˚C. The changes in MCA were measured, with the MCA of the untreated control (no metal ions) set as 100%.

Kinetic measurement for Penicillium purpurescens MCE. The kinetic parameters of fungal MCE were determined using a modified version of the method described by He *et al* (19). Casein solutions at varying concentrations (0.1 and 3.0 g/l) were used as substrates to measure the PA following the protocol described in the study by Kumar *et al* (14). The Michaelis-Menten constant (*Km*) and maximal velocity (*Vmax*) were derived from a Lineweaver-Burk double-reciprocal plot, as established by Lineweaver and Burk (20). This analysis provided insight into the catalytic efficiency and substrate affinity of the enzyme.

Statistical analysis. All experiments were performed in triplicate. All reported values are expressed as the mean ± standard deviation (SD). Statistical analysis was conducted using SPSS 17.0 (IBM Corp.). Comparisons between two groups were performed using a t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results and Discussion

Screening of different organisms for MCE production. The MCA of various fungal and bacterial strains, measured in MCA/ml is presented in Table I. Among the tested strains, only a few exhibited detectable MCA, while the majority did not exhibit any activity. *Penicillium purpurescens* demonstrated the highest MCA (190±3.4 MCA/ml), suggesting its potential as a robust MCA. *Aspergillus oryzae* under shaking conditions in PDA medium (Materials and methods) also exhibited notable activity (102±3.4 MCA/ml), indicating that cultivation conditions significantly influence enzyme production (9,21). *Curvularia lunata* DHE5 and Noh2 displayed moderate activity (60.8±2.1 and 45±1.2 MCA/ml, respectively), which may warrant further optimization for industrial applications. The absence of MCA in the majority of strains, including *Scopulariopsis brevicaulis*, *Penicillium javanicum* and various *Aspergillus* and *Penicillium* species, suggests that MCEs are strain-specific and not universally produced (22). The variability in MCA between different growth conditions (static vs. shaking, dox vs. PDA) highlights the importance of optimizing fermentation parameters for maximal enzyme yield (23).

Effect of different media on MCE production using SSF and SmF (shaking and static conditions). The results obtained (Table II) revealed variations in MCE production by *Penicillium purpurescens* across different media, aligning with findings from other fungal studies in Table I. For example, *Aspergillus oryzae* exhibited high MCE activity in wheat bran-based SSF (24), comparable to the optimal performance observed in *Penicillium purpurescens* under similar conditions. Likewise, *Thermomucor indicae-seudaticae* N31 was investigated using submerged fermentation with 4% wheat bran in a 0.3% saline solution, incubated at 45˚C and 150 rpm for 72 h (25), Furthermore, in solid-state fermentation of *Mucor racemosus*, molasses and casein were identified as significant carbon and nitrogen sources (26). Further emphasized is the role of substrate composition in fungal enzyme production, demonstrating that lignocellulosic substrates significantly influence MCE yields in SSF. The lack of clotting activity in Sabouraud and natural potato media for *Penicillium purpurescens* is consistent with reports on *Neurospora intermedia*, which also displayed low enzyme production in similar nutrient-limited substrates (27). These comparisons underscore the critical influence of media composition on fungal MCE production, with SSF often proving superior due to better fungal growth and enzyme induction, as demonstrated by *Penicillium purpurescens* and other fungi such as *Aspergillus oryzae*.

Effect of pH on Penicillium purpurescens MCE. As illustrated in Fig. 1, the pH of the culture medium significantly influenced both MCE production and PA in *Penicillium purpurescens*, with optimal activity observed at near-neutral pH (6.0-6.5). No activity was detected at highly acidic (pH 2-3) or alkaline (pH 7) conditions, while a progressive increase was noted

Table II. Effect of different media on *Penicillium purpurescens* MCE production using SSF and SmF (shaking (180 rpm) and static).

A, SmF

Medium	Milk-clotting activity MCA/ml Shaking (mean ± SD)	Static (mean ± SD)	P-value
Synthetic potato dextrose broth	0.0	0.0	-
Modified dox ($NaNO^3$+5% sucrose)	290±3	266±0.7	0.079
Yeast extract peptone dextrose	0.0	213.3±2.5	0.0001[a]
Malt broth	290±5.1	80±1.8	0.005[a]
Czapex dox	133.3±3.1	100±	0.075
Starch nitrate	100±2	260±1.9	0.0001[a]
Specific medium	0.0	290±4.5	0.0001[a]
Modified dox (5% sucrose + yeast + peptone)	-	-	-
Natural potato	-	-	-
Sabouraud	-	-	-

B, SSF

Agricultural waste	Shaking (mean ± SD)	Static (mean ± SD)	P-value
Wheat bran	320±1.3	106±2.5	0.0001[a]
Sugar cane baggase	0.0	0.0	
Banana peel	0.0	0.0	
Guava seeds	94±2.1	21±1.98	0.005[a]
Rough corn stover	0.0	0.0	
Pomegranate peel	0.0	0.0	
Tangerine peel	0.0	0.0	
Saw dust	0.0	0.0	

Values are expressed as the mean ± SD of enzyme activity as triplicates; [a]P-values ≥0.05 indicate a statistically significant difference.

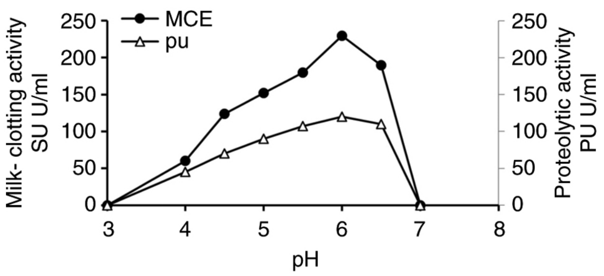

Figure 1. Effect of initial pH on milk-clotting activity of the enzyme. Enzyme activity was measured at various initial pH values ranging from (lowest pH 3) to (highest pH 8). The optimal activity was observed at pH 6, indicating the preferred operating range of the enzyme under neutral conditions. SU, Soxhlet units; PU, proteolytic units.

from pH 4 to 6, peaking at pH 6 (188 MCE units, 120 PU). This trend is in accordance with findings in other fungi, such as *Aspergillus oryzae*, which exhibited maximal MCE activity at pH 5.5-6.0 (24), and *Mucor racemosus*, which exhibited optimal production at pH 4.8 (3,26). Similarly, *Neurospora intermedia* demonstrated reduced enzyme yields at extreme pH levels, emphasizing the importance of near-neutral conditions for fungal protease stability and secretion (27). The sharp decline in *Penicillium purpurescens* activity at pH 7 suggests enzyme denaturation or impaired fungal metabolism under alkaline conditions, a phenomenon also observed in *Bacillus* spp (28), which are known to produce enzymes that remain stable across a broad pH range (6.0-10.0).

Effect of temperature on Penicillium purpurescens MCE production. The initial temperature significantly influences the production of MCE and PA in *Penicillium purpurescens*. As demonstrated in Fig. 2, the MCE and PA increased with temperature, peaking at 50˚C (145 U/ml MCE, 120 U/ml PU), followed by a sharp decline beyond 60˚C due to enzyme denaturation. Similar trends have been observed in other fungi, such as *Aspergillus oryzae* and *Rhizopus miehei*, which exhibit optimal MCE production at 30-40˚C and 45-55˚C, respectively (24,29). *Aspergillus niger* has also been shown to exhibit a reduced PA >50˚C, suggesting that thermal stability is common among fungal enzymes. These findings highlight the importance of optimizing temperature for maximal enzyme yield and stability in microbial MCE production.

Table III. Comparative MCA/PA ratios of fungal and animal rennets.

Source	MCA (SU/mg)	PA (U/mg)	MCA/PA Ratio	(Refs.)
Penicillium purpurescens	620	2.0	320	Present study
Rhizomucor miehei	450	2.2	~204	(32)
Mucor pusillus	520	3.8	~137	(25)
Calf rennet	650	1.5	~433	(46)

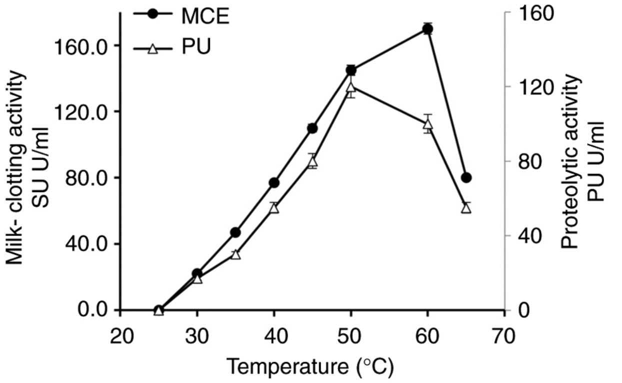

Figure 2. Effect of incubation temperature on the production of *Penicillium purpurescens* milk-clotting enzyme. SU, Soxhlet units; PU, proteolytic units.

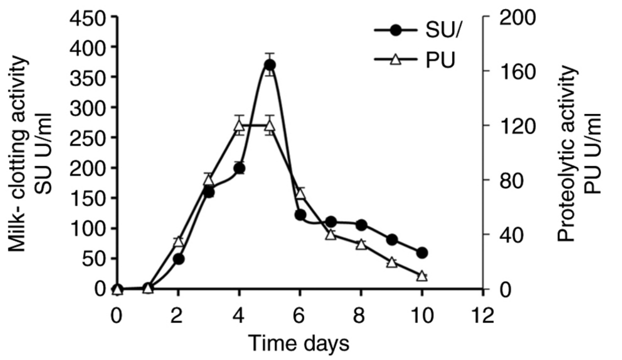

Figure 3. Time-course of *Penicillium purpurescens* growth, milk-clotting activity, and proteolytic activity in modified specific medium, production during different fermentation times (1-10 days). SU, Soxhlet units; PU, proteolytic units.

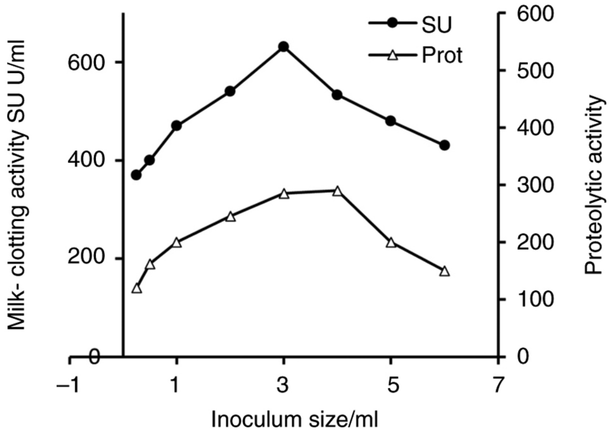

Figure 4. Effect of inoculum size on milk-clotting enzyme and protease production by *Penicillium purpurescens*. SU, Soxhlet units.

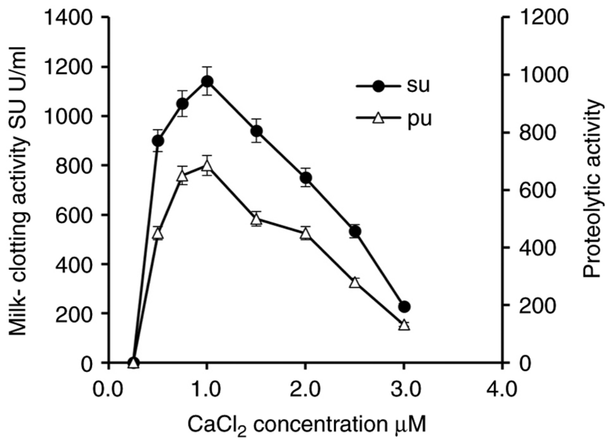

Figure 5. Effect of $CaCl_2$ concentration on milk-clotting (SU) and proteolytic (PU) activities of *Penicillium purpurescens*. Soxhlet units; PU, proteolytic units.

Fermentation time. The effect of fermentation time on *Penicillium purpurescens* MCE production and proteolytic activity follows a trend where enzyme activity initially increases with time before declining due to proteolytic degradation or nutrient depletion. As demonstrated in Fig. 3, optimal MCE production often occurs between 4-5 days, coinciding with peak fungal biomass and secondary metabolite secretion (30). Beyond this period, prolonged fermentation leads to reduced enzyme stability and increased proteolytic degradation, as observed in *Penicillium purpurescens*, where MCE activity decreased after day 4. Similar patterns have been observed in other fungi, such as *Aspergillus oryzae* and *Rhizomucor miehei*, where maximal MCE yield was achieved within 3-5 days before the activity diminishes (24,31). In the present study, the MCE demonstrated a ratio of 320, which is higher than the reported ratios for commercial counterparts and moderately close to that of calf rennet (~433), suggesting promising industrial potential, As shown in Table III, *Aspergillus niger* exhibits peak protease secretion at 72-96 h, after which activity declines due to autolysis. In contrast, the enzyme from *Thermomucor indicae-seudaticae* N31 was successfully used in the production of high-quality Prato cheese, maintaining stability without changes over 60 days (32). These findings highlight the importance of optimizing fermentation duration to balance enzyme yield and stability, which may vary across species.

Effect of inoculum size on Penicillium purpurescens MCE production. As illustrated in Fig. 4, the inoculum size

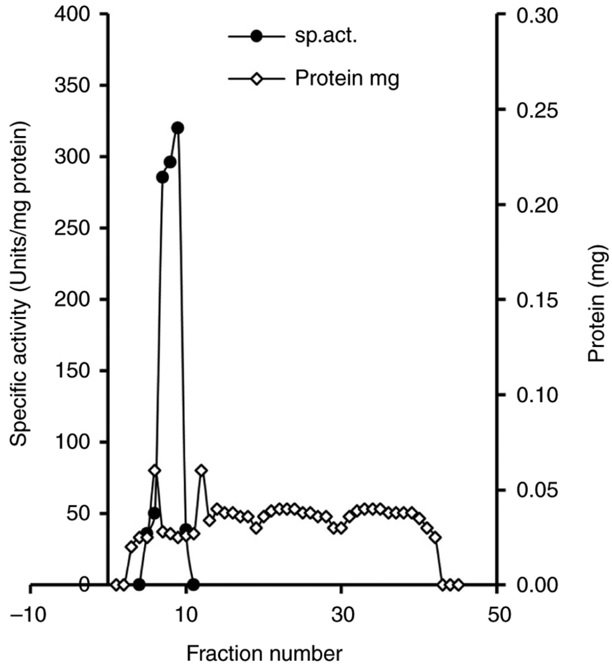

Figure 6. Elution profile of milk-clotting enzyme from *Penicillium purpurescens* purified by acetone fractionation, then DEAE-cellulose ion-exchange chromatography. Protein content was monitored at 280 nm, and milk-clotting activity was assayed in collected fractions. Active MCE fractions were observed at (NaCl concentration range] 0.05 to 0.1 mM), indicating successful separation from non-active proteins.

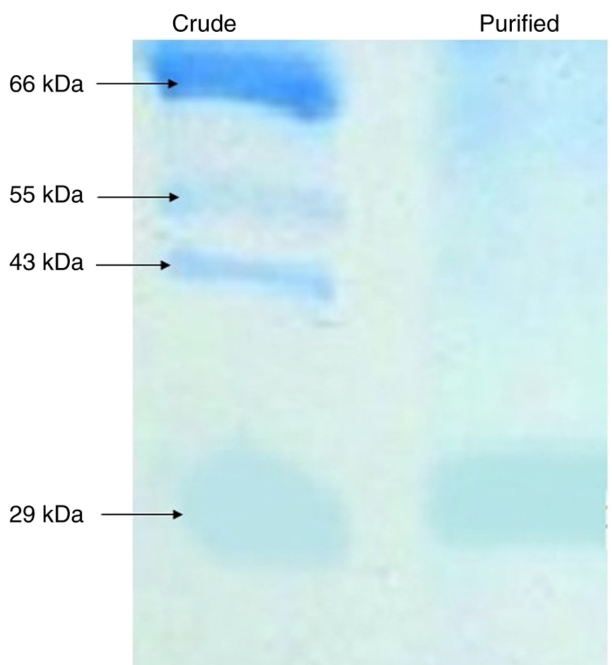

Figure 7. SDS-PAGE analysis of enzyme extract. Lane 1 (crude): Protein molecular weight marker (kDa); Lane 2 (purified): Purified milk-clotting enzyme from *Penicillium purpurescens*.

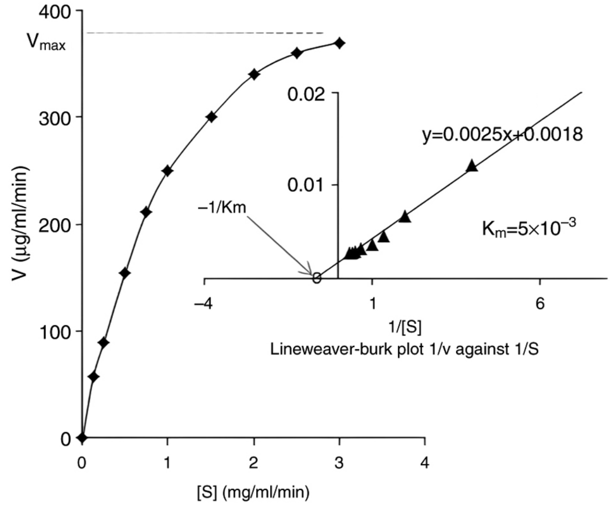

Figure 8. Michaelis-Menten and Lineweaver-Burk plot of reaction velocity vs. casein concentration for the milk-clotting enzyme. V, velocity; *V*m, maximal velocity; S, casein substrate concentration; *K*m, Michaelis constant=5x10^{-2} M.

significantly influenced the production of MCE and PA in *Penicillium purpurescens*. Research has shown that increasing the inoculum size from 0.25 to 3 ml enhances both enzyme activity (from 370 to 631 SU) and proteolytic activity (from 120 to 285 U/ml), likely due to improved fungal biomass and nutrient utilization (33). However, beyond 3 ml, a decline in MCE activity (533 SU at 4 ml) and PA (290 U/ml), occurs, possibly due to nutrient depletion or metabolic stress. Similar trends have been observed in other fungi, such as *Aspergillus oryzae*, and *Aspergillus clavatus*, where optimal inoculum size (1-2x10^6 spores/ml) maximizes protease production, while higher densities reduce yields due to oxygen limitation (24,33). Likewise, *Rhizopus microsporus* exhibits peak MCE activity at 5% (v/v) inoculum, with declines at higher concentrations (34,35). These findings suggest that inoculum optimization is critical for balancing microbial growth and enzyme synthesis across fungal species.

Effect of $CaCl_2$ on Penicillium purpurescens MCE. As demonstrated in Fig. 5, MCA peaked at 1% $CaCl_2$ (1,140 U/ml), while PA reached its maximum level (685 U/ml) at the same concentration. Higher $CaCl_2$ levels (>1%) lead to a decline in both MCA and PA, suggesting enzyme inhibition or destabilization at an elevated ionic strength. Similar trends have been observed in other fungi, such as *Bacillus velezensis* DB219, where optimal MCE production occurred at 0.5-1% $CaCl_2$ beyond which activity decreased due to possible metal ion interference with enzyme stability (36). *Rhizopus miehei* also exhibited reduced proteolytic activity at $CaCl_2$ concentrations above 1.5%, attributed to conformational changes in the enzyme (37). These findings highlight the critical role of Ca^{2+} in modulating fungal MCE activity, with species-specific optimal thresholds. The decline in *Penicillium purpurescens* MCE beyond 1% $CaCl_2$ aligns with previous research on *Neurospora crassa*, where excessive Ca^{2+} disrupted secretion pathways (38) underscoring the delicate balance between cation stimulation and inhibition in fungal enzyme systems.

MCE preparation and purification. As illustrated in Fig. 6, the purification of the MCE from *Penicillium purpurescens* involves fractionation steps that enhance specific activity, as evidenced by the data exhibiting increased activity in fractions 7-9, with peak specific activities of 286. 296 and 320 U/mg

Table IV. Effect of metal ions on *Penicillium purpurescens* MCE activity.

Metal ion	Concentration (μM)	Relative activity (%)
Ca^{2+}	10	110
Ca^{2+}	50	150
Mg^{2+}	50	120
Cu^{2+}	10	40
Hg^{2+}	10	10
Na^{+}	10	95

Table V. Physicochemical and sensory properties of curd formed by *Penicillium purpurescens* milk-clotting enzyme.

Parameter	Observation
Curd yield (%)	37% fresh weight
Dry matter (%)	45-50%
Firmness	High, non-fragile,
Coagulation time	1-5 min
pH at coagulation	6.0-6.5
Bitterness	None
Odor	Clean, milky
Syneresis	Low to moderate

U/mg, respectively. These fractions likely contain the purified enzyme, as indicated by their high specific activities compared to earlier fractions with negligible activity. Similar purification trends have been observed in other fungal species, such as *Aspergillus oryzae* and *Rhizomucor miehei*, also produce proteases with milk-clotting properties (24,37). For instance, *Rhizomucor miehei* yields a highly active aspartic protease (rennin-like enzyme) after purification, with specific activities comparable to those of *P. purpurescens* (37). However, some fractions (e.g., 10-12) exhibit variability, possibly due to contamination or partial denaturation. Further purification steps, such as ion-exchange or gel-filtration chromatography, could improve homogeneity, as demonstrated in *Aspergillus niger* protease purification (23). These data suggest that *Penicillium purpurescens* is a promising source of milk-clotting enzymes, with purification efficiency comparable to other industrially relevant fungi.

Molecular weight of Penicillium purpurescens MCE. The MCE from *Penicillium purpurescens* has a molecular weight of ~29 kDa (Fig. 7), which is comparable to other fungal aspartic proteases used in cheese production. For instance, *Rhizomucor miehei* produces a 38.6-kDa aspartic protease (rennet) (37), while *Aspergillus oryzae* secretes a 43-kDa MCE (39). Similarly, *Endothia parasitica* yields a 33.8 kDa protease (40). The lower molecular weight of *Penicillium purpurescens* enzyme suggests structural differences that may influence substrate specificity and thermal stability. These fungal enzymes are preferred over animal rennet due to their cost-effectiveness and suitability for vegetarian cheese production. Further comparative studies on their biochemical properties could optimize their industrial applications.

Effect of pH and temperature on cheese quality. The MCA of the enzyme produced by *Penicillium purpurescens* exhibited a distinct dependence on pH, with optimal activity observed at pH 6, as shown in Fig. 1, where the enzyme reached a maximum activity of 336 MCE/ml. This result suggests that the enzyme functions best under mildly acidic to neutral conditions, aligning with observations from other microbial MCEs. For instance, similar pH optima have been reported for enzymes derived from *Mucor pusillus* and *Rhizomucor miehei*, which also exhibit peak MCA around pH 5.5-6.5 (37,41). The activity was significantly lower at both extreme acidic (pH 3, 120 MCE/ml) and alkaline (pH 10, 200 MCE/ml) conditions, indicating a narrow optimal pH range for maximum proteolytic efficiency. Fungal milk-clotting enzymes, as all enzymes, are sensitive to pH; deviations from their optimal pH range can lead to decreased stability and a reduced ability to bind to their substrate, milk proteins. This phenomenon underscores the critical need to control pH during dairy processing to ensure optimal enzyme performance, which directly impacts the efficiency and quality of products such as cheese (42).

The MCE derived from *Penicillium purpurescens* exhibited a pronounced temperature-dependent activity profile. Enzyme activity increased steadily from 30˚C, reaching its maximum at 50˚C with an activity of 360 U/ml, which indicates this temperature as optimal for catalytic function, as shown in Fig. 2. A sharp decline in activity was observed at 55˚C, suggesting the onset of thermal denaturation; however, the enzyme retained partial activity at 60˚C. Beyond this temperature, activity decreased markedly, with complete inactivation occurring at 90˚C. This thermal behavior is consistent with MCEs from other fungal species, such as *Rhizomucor miehei* and *Mucor pusillus*, which generally show optimal activity within the 45-55˚C range and lose enzymatic function at elevated temperatures due to structural instability (37,41). These observations underscore the critical need for precise temperature control when employing fungal MCEs such as MCE from *Penicillium purpurescens* in industrial processes such as cheese manufacturing.

Effect of metal ions on Penicillium purpurescens MCE activity. As demonstrated in Table IV, a marked influence of various metal ions was observed on the MCE activity of *Penicillium purpurescens*. The data revealed that Ca^{2+} (50 μM) enhanced enzyme activity by 150%, suggesting a potential stabilizing or activating role, while Mg^{2+} (50 μM) also promoted activity (120%), albeit to a lesser extent. By contrast, Cu^{2+} (10 μM) and Hg^{2+} (10 μM) markedly inhibited MCE function, reducing activity to 40 and 10%, respectively, likely due to metal-induced protein denaturation or active-site interference (43). The negligible effect of Na^{+} (10 μM; 95% activity) implies that monovalent ions may have minimal impact on this enzyme system. These findings align with recent studies highlighting the dual role of metal ions as either cofactors or inhibitors in fungal MCE kinetics (44).

Kinetic parameters of Penicillium purpurescens MCE. The Michaelis constant (K_m) of the MCE from *Penicillium purpurescens* was determined to be $5x10^{-2}$ M (Fig. 8), indicating its moderate affinity for the substrate compared to other fungal proteases. For instance, *Aspergillus oryzae* produces a milk-clotting enzyme with a lower Km ($2.5x10^{-2}$ M), suggesting higher substrate affinity, while *Rhizomucor miehei* exhibits a markedly lower K_m ($3x10^{-3}$ M), reflecting superior catalytic efficiency in cheese-making (37,44). By contrast, *Endothia parasitica* has a higher Km ($12x10^{-1}$ M), indicating weaker substrate binding (40). The K_m value of *Penicillium purpurescens* positions it as an intermediate among fungal coagulants, potentially offering balanced proteolytic activity for dairy applications.

In the authors' laboratory, the solid-state fermentation process described herein demonstrated good reproducibility, as repeated batches produced under identical conditions consistently yielded comparable enzyme activity and quantity. This reproducibility at the laboratory scale supports the robustness of the optimized process. However, the scalability of the method has not yet been assessed due to some limitations we faced, including the lack of a pilot at the current time, a lack of funds, and the absence of an experienced team to deal with a pilot and its maintenance. Future research is required to focus on pilot-scale trials to evaluate the feasibility of large-scale enzyme production. Scaling up SSF may present challenges, including maintaining uniform moisture distribution, temperature control and adequate aeration in larger bioreactors, which need to be addressed to ensure consistent enzyme quality and yield in industrial applications (45).

Based on the results presented herein, the MCE from *Penicillium purpurescens* produces high-quality cheese curds with optimal characteristics, including curd yield, firmness and sensory properties (Table V). Further comparative studies are required to optimize its use in industrial processes. Collectively, *Penicillium purpurescens* demonstrates strong potential as a microbial rennet source, with performance comparable to established fungal producers. Further optimization and industrial-scale validation could enhance its applicability in cheese manufacturing.

The present study had certain limitations which should be mentioned. The activity of the enzyme against α- and β-casein fractions could not be assessed due to logistical challenges beyond the authors' control, including delays in chemical imports.

In conclusion, the present study highlights *Penicillium purpurescens* as a highly promising fungal strain for MCE production, demonstrating superior clotting efficiency compared to other screened strains. Optimal MCE production was achieved under SSF with wheat bran, near-neutral pH (6.0-6.5), and moderate temperatures (50˚C), with fermentation kinetics peaking at 4-5 days. The enzyme exhibited enhanced activity at 1% $CaCs_2$, but declined at higher concentrations, suggesting ionic strength sensitivity. Purification yielded fractions with high specific activity (up to 320 U/mg), and the molecular weight of the enzyme (29 kDa) and kinetic properties (K_m=$5x10^{-2}$ M) position it as a competitive alternative to commercial fungal rennets such as those and *Aspergillus oryzae*. These findings underscore *Penicillium purpurescens* as a viable candidate for industrial cheese production, though further scale-up and stability studies are needed to confirm its commercial applicability. Future research is warranted.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

THA, LAM, EMA and DHEG performed the screened for MCE in different strains, enzyme production form selected strain, enzyme characterization, purification and biochemical enzyme kinetics. SF performed the electrophoresis, and assisted in the drafting of the manuscript. THA wrote the manuscript. THA and LAM confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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