

Separation, Purification, and Phylogenetic Characterization of Detergent-compatible Protease Produced by *Penicillium* Species

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Abstract

Background: Filamentous fungal forms are said to secrete multiple peptidases with portent detergent capabilities in addition to their usage in food, beverages, and pharmaceutical industries. **Objective:** The current study isolated and purified fungal isolates from clinical samples and studied their capability as caseinolytic and in removing blood stains from fabrics. **Materials and Methods:** The present work isolated nine isolates, which are protease-positive and out of them eight belong to *Penicillium* sp. From the IS2 region, amplification of the isolates concluded their match using Blastn as *Penicillium citrinum* in majority. **Results:** The enzyme extract (P18), which was found effective in removing blood stains with good caseinolytic activity, was found to belong to *P. citrinum* as from Blastn studies. In addition, these proteases extracted were highly compatible with commercially available detergents. P4 was found to remove blood stains from fabrics in less than 30 min ($P < 0.02$) when compared with other extracts (P1 and P4). **Conclusion:** The ability to secrete protease in promising amounts along with its potential usage as detergent makes these enzymes useful in industries, especially for the laundry industry.

Keywords: Blood stain removal, caseinolytic activity, ITS2 sequencing, *Penicillium* sp., protease

INTRODUCTION

Due to its quality, workforce, and political stability, economic potential textile sectors play a significant role in a country's economy. The textile industry is therefore ever increasing its demand for cleanliness, which causes minimal or no pollution. Since several centuries ago, enzymes have been used in textile manufacturing as a replacement for conventional chemicals in a number of processes. This method, among all tested methods, is the most effective for removing old, resistant adhesives and stains.^[1] A separate benefit of enzymes is that the enzymes speed up the degradation process of the constituent components of pastes, thus allowing them to be removed more effectively from extremely thick accretions that require too long and repeated washing.^[2,3]

The protease action, however, results in severe damage to natural protein fibers, such as silk and wool, when these fibers are washed with detergents containing proteases.^[4]

In recent years, a promising and low-cost biotechnology, solid substrate fermentation (SSF), has emerged. Various filamentous fungi have been used to develop highly efficient SSF-based processes such as biopulping, waste decomposition, pharmaceuticals, enzymes, pesticides, biosurfactants, and plant growth factor production.^[5] They have considerable industrial potential due to their biochemical diversity and a wide variety of applications such as food and tannery products, medical formulations, detergents, waste treatment, silver recovery, and amino acid mixtures resolution.^[6]

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As the most significant group of industrial enzymes, proteases carry out numerous functions and find commercial application in a variety of industries.^[7] Applying these polymers in detergents, food, pharmaceuticals, and leather has wide spread applications.^[8] Proteins have been successfully used as an alternative to chemicals and as an eco-friendly indicator of the environment.^[9,10] Globally, enzyme market sales are dominated by proteases, which account for approximately 60% of the market.^[11] In general, proteases are synthesized and produced by several microorganisms such as bacteria, fungi, and actinomycetes,^[12] out of which fungi are exclusively known to produce extracellular enzymes. *Aspergillus*,^[13] *Rhizopus*, *Mucor*, and *Penicillium*^[14] are among the genera known to produce proteases.

The removal of proteinaceous material from the stains becomes easier with the use of protease-based detergent preparations.^[15] Most commercial detergents used today are serine proteases produced by strains of *Bacillus*. However, fungal alkaline proteases have the advantage of being easily converted into microbial-free enzymes.^[16] The detergent industry has recently become more interested in using fungal proteases. Recently, there has been increasing research about utilization of fungal proteases in the detergent industry.^[17]

The use of fungal strains for commercial purposes is, however, limited. *Penicillium* species were evaluated for their ability to produce extracellular protease under optimal conditions in this work. The present study reports identifying, isolating, and purifying thermo-stable alkaline proteases from the aforementioned bacterial species.

To determine their inherent capability in producing the enzyme of interests, the study sequenced the genes of the strains at the genus level using 16srRNA sequencing. Blood stains were also used in stain removal studies, along with commercial detergents to determine the effectiveness of stain removal. This protein is an ideal candidate for an ecofriendly detergent-based formulation, according to our data.

MATERIALS AND METHODS

Isolation of fungal strains

Rotten fish waste was collected from the local fisheries market yard in a sterile container. Fish gills, scales, fins, gut, and muscle tissue were collected separately and suspended into 100 mL of sterile distilled water. About 2 mL of sample from each sample suspension was used for serial dilution and plated onto potato dextrose agar (PDA) at 24°C. The mycelial mats thus obtained were screened for their alkaline protease-producing capability, as described by Rodrigues.^[18] The colonies on the agar plates were effused, greenish blue, and hairy. In brief, each colony was then spotted onto skimmed milk agar (skimmed milk powder 2%, PDA 5%, pH 8) and incubated at 24°C for

about 3–5 days.^[19] Colonies with clear hydrolytic zone were further selected as protease-positive and used in the production process.^[20]

Protease production and extraction

For producing the enzyme, mycelia plugs about 3–5 mm Ø from a 3-day-old PDA culture were transferred to 200 mL of production medium (KCl 1.0 g/L, KH_2PO_4 6.7 g/L, K_2HPO_4 14.3 g/L, MgSO_4 0.5 g/L, NaNO_3 4.3 g/L, $(\text{NH}_4)_2\text{SO}_4$ 1.4 g/L, yeast extract 2.0 g/L, 2% Soya, and 1 mL of oligo elements) and grown at 24°C for 3–5 days. The medium was maintained at a pH of 6.5 and throughout the fermentation, agitation was provided at 180 rpm. After the fermentation period, the medium was filtered and used for the extraction process. Proteinase was extracted from the crude filtrate as described.^[21]

About 100 mL of Tween-80 (1% in water) was added to the fermented medium and homogenized thoroughly at 150 rpm on a rotary shaker for 2 h. Following homogenization, the contents were filtered and centrifuged (8000 rpm for 20 min) at 4°C. The filtrate obtained contains the crude enzyme and further purified using the ammonium sulfate method.^[22]

Enzyme purification and concentration

Enzyme was precipitated by the ammonium sulfate method and dialyzed to obtain the concentrated form. The crude filtrate obtained in the previous section was initially saturated with 30% of ammonium sulfate and centrifuged at a maximum speed for 15 min at 4°C. The pellet obtained was then re-suspended in about 10 mL of phosphate buffer (0.1 M, pH 7). To the suspension, 80% of the saturated ammonium sulfate (52.5 g) was added slowly with constant stirring and centrifuged at a maximum speed for 10 min. The pellet obtained was re-suspended in 2 mL of phosphate buffer^[23] and used for the protease assays. The purified extract was further concentrated by dialysis using the cellulose acetate dialysis tube, and the concentrated enzyme was then used for further estimation assays.^[24]

Estimation of protease activity

Protease activity of the enzyme was estimated as described^[25] but with a slight deviation. To 5 mL of the substrate (1% Casein in 200 mM glycine-NaOH buffer; pH 10.0), varying volumes of the enzyme at different concentrations (20, 40, 60, 80, and 100 µg/mL) were added and incubated at 37°C for 20 min. An aliquot of 1 mL of trichloroacetic acid (TCA) (110 mM) was added to stop the reaction and further incubated at 37°C for 30 min. After incubation, 2 mL of the sample was collected into a fresh tube and added with 5 mL of sodium carbonate and 1 mL of Folin–Ciocalteu's reagent (FC 1:1 in water). The contents were mixed thoroughly and incubated again at 37°C for 30 min and recorded for absorbance at

660 nm. Tyrosine (0–100 µg/mL) was used as standard in the study. The amount of L-tyrosine released from the substrate was quantified at 680 nm. The more the tyrosine released, greater is the protease activity. Protease activity [U/mL] = (µmoles of tyrosine equivalents released × Total volume of assay [mL] × Dilution factor)/(Total volume of enzyme used in the assay [mL] × Time of assay [min] × Volume in cuvette [mL]).

Caseinolytic activity: Caseinolytic activity was estimated as described by Singh *et al.*^[26] with slight modifications. In brief, about 0.5 mL of casein was added with varying concentrations of 20–100 µg/mL of the purified enzyme and incubated at 37°C for 2 h. Then 1.5 mL of TCA (0.44 M) was added to stop the reaction, and the contents were allowed to stand for 10 min at room temperature before centrifuging at 2000 rpm for 15 min. The supernatant obtained was added to 2 mL of sodium carbonate (0.4 M) and 0.5 mL of Folin–Ciocalteu reagent (1:2 in water). Absorbance was recorded at 660 nm using a spectrophotometer. Trypsin (40 µg/mL) was used as a positive control in the study. One unit of enzyme activity was defined as the amount of the enzyme required to increase the absorbance of 0.01 at 660 nm/h at 37°C.

Extraction of DNA from fungal mats

Genomic DNA was extracted from the fungal samples by the CTAB-phenol-chloroform-isoamyl alcohol method.^[27] In brief, about 0.2 g of lyophilized mycelium was homogenized with mortar and pestle using 500 µL of extraction buffer (200 mM Tris–HCl (pH 8.0), 25 mM EDTA (pH 8.0), 250 M NaCl, 10% CTAB) according to Spadoni *et al.*^[27] The contents are transferred to a fresh tube and added to 3 µL of proteinase K and 3 µL of RNase and vortexed. After 1 h of incubation at 37°C, the tubes were incubated at 65°C for 10 min in a water bath. About 100 µL of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the mixture and centrifuged at 10,000 rpm for 10 min. The clear upper aqueous phase was recovered with chloroform: isoamyl alcohol (24:1) and spun down at a maximum speed for 5 min to recover the aqueous phase. The DNA is now precipitated with ice-cold isopropanol and stored at –20°C.

ITS2 sequencing and phylogeny

Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS2) regions was done with specific primers ITS86-F (5′-GTGAATCATCGAATCTTTGAAC-3′) and ITS4-R (5′-TCCTCCGCTTATTGATATGC-3′),^[28] which could yield a fragment of approximately 200–400 bp. The nine samples which are positive for protease were used as DNA templates. In addition, DNA of *Escherichia coli* and *Drosophila melanogaster* was also used to confirm the specificity of the primers to fungal species. PCR was performed with initial denaturation at 93°C for 10 min, and 35 cycles of 45 s at

93°C, 60 s at 55°C, and 50 s at 72°C. The PCR products were then resolved on 1.2% agarose gel electrophoresis, and aliquots were sent for sequencing.

The sequence obtained was then searched against database for identification of the nearest related strains and aligned using the BioEdit software.^[29] The DNA sequences obtained were then analyzed for homology using the BLASTN program with a similarity of ≥99% to ≥97%.^[30] In the current study, MEGA 7 (v. 7.2) was used in deducing the phylogenetic relation,^[31] and the trees were constructed using neighbor-joining^[32] methods (bootstrap 1000). The Jones–Taylor–Thornton model was used in both the cases with a site coverage cut-off at 95%.^[33]

Compatibility with commercial detergents

The purified enzyme was studied for its compatibility with other commercially available detergents such as Surf Excel, Rin, Tide, and Ariel. In brief, about 100 µL of enzyme extract was added to 2 mL of the detergent (2%) and maintained at pH 7.5. The contents were incubated overnight at room temperature and enzyme activity was measured as mentioned in the previous section. Samples without any detergent were considered as 100% protease active. Only samples P1, P4, and P18 were used in the study owing to their high protease activity.

Stain removal evaluation

The purified enzyme was studied for its ability to remove blood stains from fabrics. In brief, blood stains were marked onto cotton cloth pieces (10×10 cm) and placed in small troughs filled with respective solutions. Distilled water served as control. Commercial detergents such as Ariel and Rin (1%) were used as positive controls. Enzyme (0.1 mg/mL) was used as sample in the study. The troughs were then incubated at 40°C for about 30–60 min and evaluated every 10 min to screen for the stain removal ability.

Statistical analysis

The data from the current study were calculated and analyzed on GraphPad Prism (CA, USA). All the data were considered statistically significant at $P < 0.05$ and $P < 0.02$. All the experiments were done in triplicates and expressed as average ± standard deviation (SD).

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients' verbal and analytical approval before the sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local Ethics Committee according to the verbal ethical approval on September 29, 2022.

RESULTS

Fungal isolation and screening for hydrolytic species

Among nine colonies, our study found that eight of them belonged to *Penicillium* sp. and one of them to *Aspergillus* species. Among the eight selected strains, P18 was found to show clear hydrolytic zone with a significant diameter of 2.56 followed by P1 (2.13) and P4 (2.11) [Figure 1].

Protease and caseinolytic activity

Among the eight strains which are found to be positive for protease activity, P18 showed significantly high amounts of enzyme activity with 223 ± 2.11 U/mL followed by P1 (178 ± 3.81 U/mL) and P4 (163 ± 3.11 U/mL) [Figure 2]. P1, P4, and P18 are strongly positive for caseinolytic

activity with 2% casein as substrate. The activity was found to be increasing progressively from 20 to 100 μ g/mL of the protein extracts [Figure 3].

PCR amplification of ITS2 and phylogenetic analysis

Electropherogram of the PCR products amplified from the ITS2 region showed distinct bands approximately between 350 and 450 bp [Figure 4]. *Aspergillus* DNA template showed a distinct band at 250 bp. EC and DM showed no banding, confirming the specificity of the primers toward fungal species. These results are in accordance to the morphological analysis. Sequencing results showed that eight of them belong to *Penicillium* and 1 of them belongs to *Aspergillus* [Figure 5]. All the sequences were searched against GenBank database using

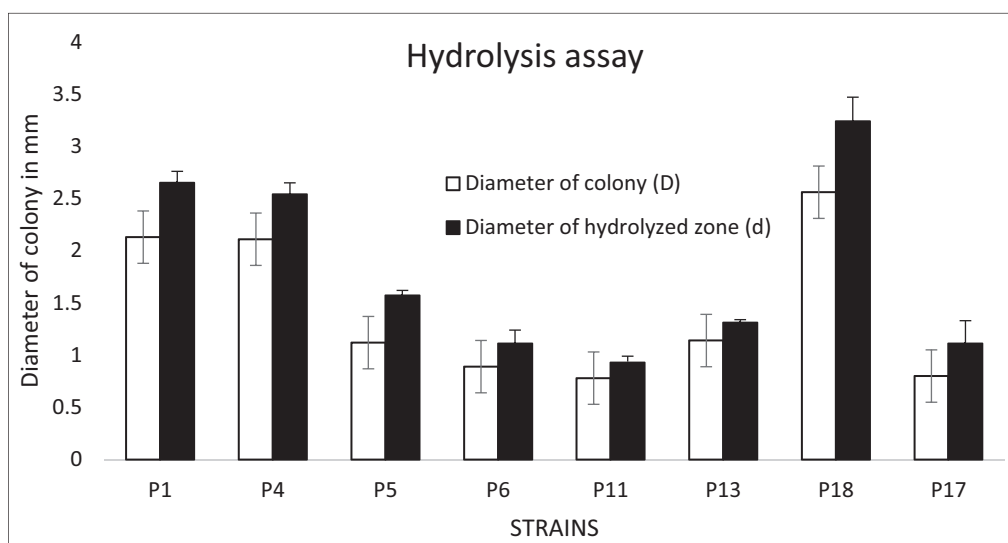


Figure 1: Hydrolytic activity of strains isolated from samples. All the strains belongs to *Penicillium* sp. All the values are average of triplicates and represented as value $\pm$ SD

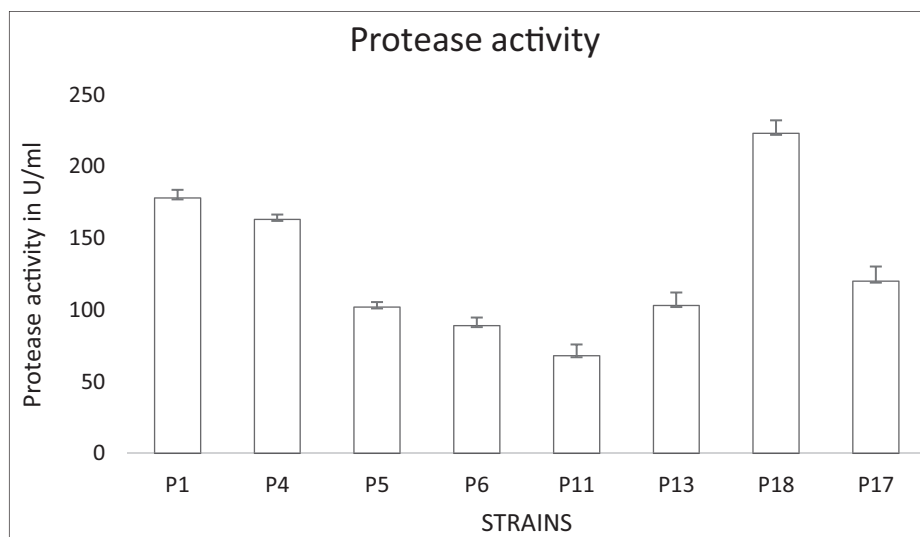


Figure 2: Protease activity of strains isolated from samples. All the strains belong to *Penicillium* sp. All the values are average of triplicates and represented as value $\pm$ SD. Values are expressed in U/mL

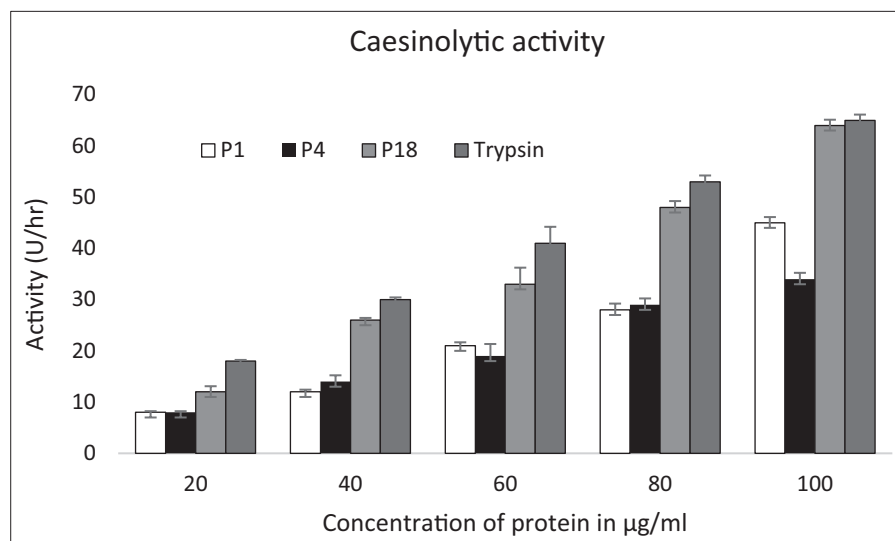


Figure 3: Caseinolytic activity of strains isolated from samples. P1, P4, and P18 are only used in the study. All the strains belong to *Penicillium* sp. All the values are average of triplicates and represented as value $\pm$ SD. Values are expressed in U/mL

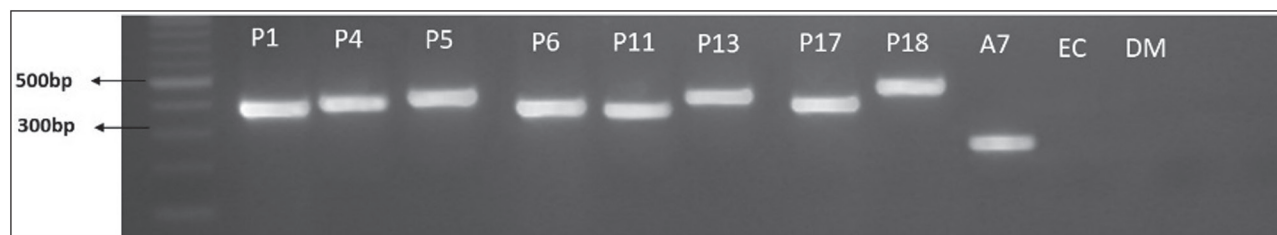


Figure 4: PCR products of fungal ITS2 on 1.2% agarose gel electrophoresis. P1, P4, P5, P6, P11, P13, P17, and P18: DNA of *Penicillium* species. A7: DNA of *Aspergillus* species. EC: DNA of *E. coli*, DM: DNA of *Drosophila melanogaster*. 100 bp DNA ladder is used as a molecular marker

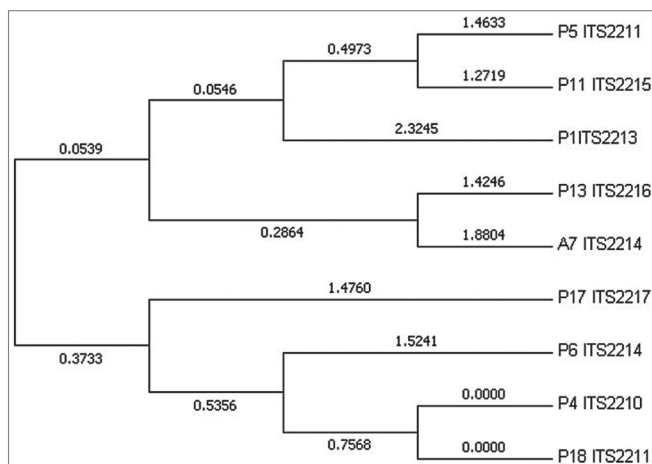


Figure 5: Phylogeny tree of the PCR amplicons based on the ITS region using the neighbor-joining method. The distances were calculated using the maximum composite likelihood method and analysis involved nine nucleotide sequences. All the gaps and missing data were removed and the final dataset contained about 232 positions. Phylogenetic analysis was done with MEGA7^[34]

BLAST (National Centre for Biotechnology Information; NCBI), in which all of the samples showed 90–95% similarity to *Penicillium* and *Aspergillus* [Table 1].

Detergent compatibility

All of the three enzymes were found to be compatible with the detergents used in the study. However, these results too confirm the strong protease activity of P18 ($P < 0.02$) than the other two enzymes (P1 and P4), even though they are compatible with the detergents ($P < 0.05$). P18 showed a significantly high activity, with 203 ± 2.11 , 180 ± 0.92 , 183 ± 1.31 , and 178 ± 1.06 U/mL, respectively, for Surf Excel, Rin, Tide, and Ariel [Figure 6]. All the detergents used are equally compatible with these enzymes ($P < 0.05$).

Blood stain removal evaluation

The de-staining property of the protease enzymes extracted from the three strains (P1, P4, and P18) was assessed on fabrics stained artificially with a drop of blood [Figure 7].

On treatment for 1 h, the blood stain was found to be removed from the fabrics with all three enzymes; however, P18 showed very high destaining ability compared with the other two (P1 and P4) enzymes. P18 was found to destain completely in 25 min, but P1 though could completely remove the stain was found to be effective at 40 min of incubation [Figure 7]. The enzymes are used along with commercial detergents such as Surf Excel and Ariel. Both of them exhibited similar results (results of Surf Excel

Table 1: Accession numbers and nearest matching strain with percent similarity

S. no.	Label	Strain name	Accession number	BLAST	
				E-value	%ID
1	P1	<i>P. citrinum</i> clone SF_138	MT529414.1	1.00E-210	96
2	P4	<i>P. citrinum</i> W51_1	MK805468.1	1.00E-131	97
3	P5	<i>P. hetheringtonii</i> strain XQ34	KU216736.1	2.00E-132	92
4	P6	<i>P. oxalicum</i> isolate ZC-7	MT410702.1	5.00E-118	91
5	P11	<i>P. citrinum</i> UH.1552.182	MZ374598.1	4.00E-133	96
6	P13	<i>P. citrinum</i> C42	MT875318.1	3.00E-192	92
7	P17	<i>P. griseofulvum</i> RIZ10.3	MW260117.1	5.00E-89	93
8	P18	<i>P. citrinum</i> AKF2-KU	MN879404.1	1.00E-121	97
9	A7	<i>niger</i>	AM269999.1	9.00E-171	92

%ID: percent identity, BLAST: Basic Local Alignment Search Tool

Data collected from Blastn (NCBI Blast:emb|AM745115.1| (nih.gov))

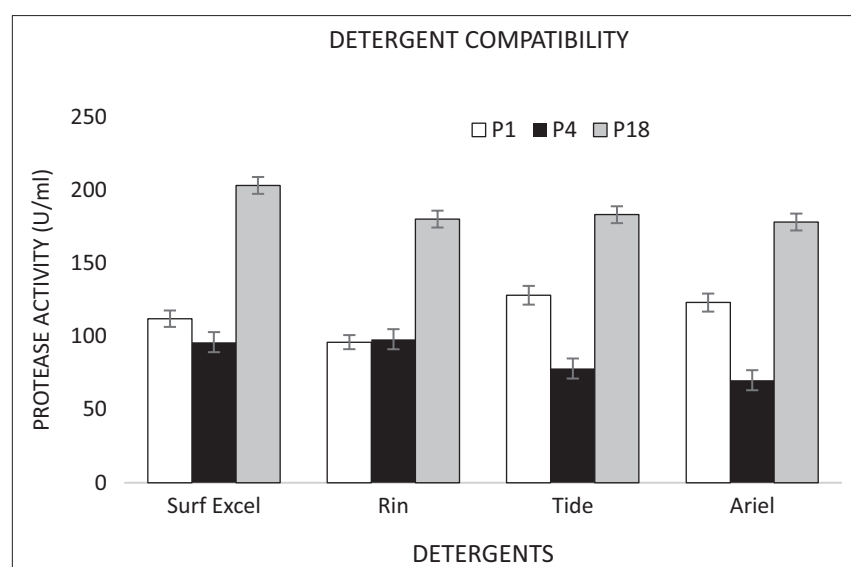


Figure 6: Detergent compatibility of protease enzymes extracted from the selected strains along with commercial detergents such as Surf Excel, Rin, Tide, and Ariel. Protease activity was expressed in U/mL and is average of triplicates. Data represented in mean $\pm$ SD

were shown). These results are again in accordance to the previous protease activity results.

DISCUSSION

Penicillium chrysogenum (Pg222) was found to secrete alkaline protease with the significant hydrolytic zone from a dry-cured ham.^[35] Similarly, serine protease isolated from *P. charlesii* filtrates was found to produce significantly greater amounts of protease after 16 days of incubation.^[36,37] Reports also^[38] confirmed the caseinolytic activity of fungal strain CLII at a higher rate. Several reports also confirmed the activity by *Pleurotus* spp., which is white rot fungus with more than 30 species.^[38] Proteinases extracted from *Paecilomyces marquandii* and *Rhizopus microsporus* also showed relatively high caseinolytic activity.^[38] *Penicillium* species are found to be great in secreting both acid and alkaline proteases, which are used in various biotechnological applications. Proteases

released *P. camemberti*, *P. citrinum*, *P. griseoroseum*, *P. restrictum*, and *P. roqueforti*, all are found to be highly compatible with commercially available detergents. Present findings are in accordance with the reports of Koul and Farooq,^[39] which confirmed the potential ability of protease from *P. griseoroseum* IH-02 to be used along with detergents. Hence, our work could conclude that purified enzymes can be used along with detergents to efficiently enhance the stain removal ability of these detergents or with any other additives in laundry and textile industries. The current results are in accordance with the reports which confirmed blood stain removal ability from cotton cloth pieces using enzymes extracted from *Aspergillus flavus* and *A. fumigates*.^[40] Similar results were published, stating that blood stains could be removed with protease enzymes extracted from *A. brasiliensis*.^[41-43] The present work is in complete agreement with the reports, which states the blood de-staining ability of the protease from *P. chrysogenum* sp.^[34]

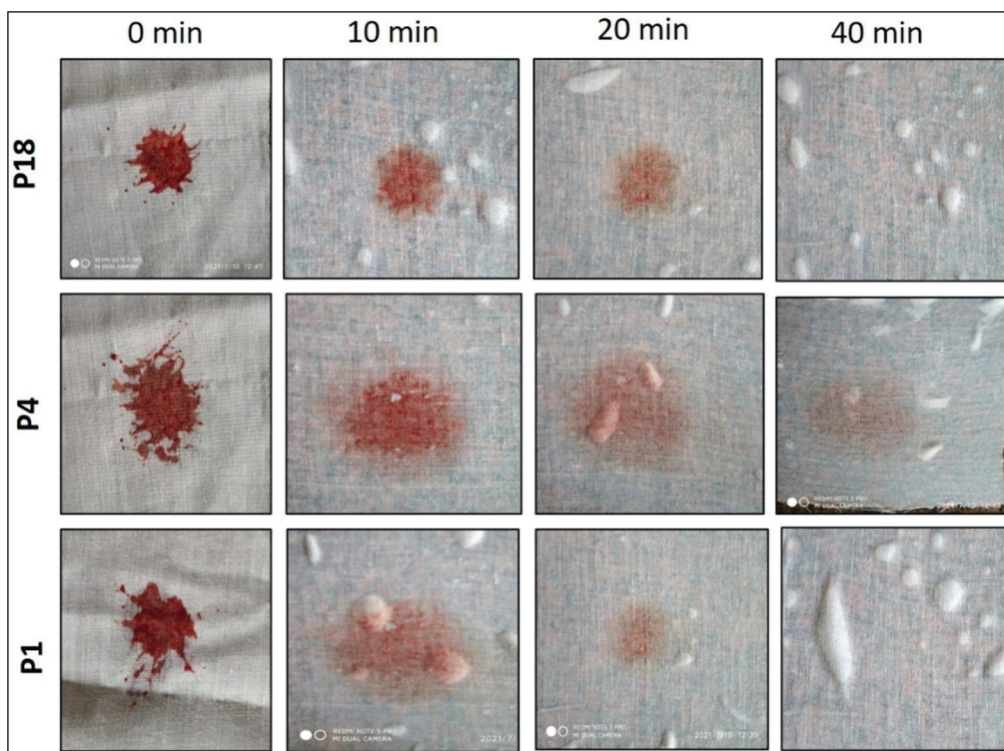


Figure 7: Fabrics displaying the effect of enzymes (P1, P4, and P18) in removing the blood stains. The enzyme extract along with the commercial detergent such as Surf Excel was used for the treatment. Ariel also showed similar results (not shown in the image)

CONCLUSION

Applying proteases in various industries has been pervasive in the recent decades, especially in biotechnology-based laundry and textile industries. Owing to its aggressive usage, hunt for microbial sources is still going, which could secrete potent and more efficient enzymes. Even though many commercial detergents and chemicals are used and synthesized, microbial-based enzymes are highly preferred owing to their vigorous growth rate. Moreover, genetic manipulation could come in handy when handling these microbes. These proteases are used in food industries to process the food, beverage and confectionaries, fodder industry, leather, and laundry industry. Considering its vast applications, proteases are being screened from filamentous fungal forms such as *Aspergillus* and *Penicillium*. We could successfully isolate, purify, and evaluate the protease activity of the protease enzyme from *Penicillium* sp.

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Conflicts of interest

The author has declared that no conflicts of interest exist.

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