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Trametes versicolor (L.) Lloyd as a source of thermostable serine protease: production and characterization

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Proteases are ubiquitously present and are among the largest groups of commercially important enzymes. Here, we investigated a wood-rot basidiomycete *Trametes versicolor* (L.) Lloyd [Syn. *Coriolus versicolor* (L.) Quél.; *Polyporus versicolor* (L.) Fr.] as a source of the enzyme serine protease, its production, and optimized to obtain a higher yield of the enzyme.. The significant variables with optimized values for maximum production of the enzyme were temperature (30°C), incubation time (120 h) and wheat bran (10 g). The yield increased by 30.76% by statistically optimizing the media. The optimized temperature and pH for the maximum protease activity was 50°C and pH 7.0, respectively. The enzyme was purified through ion exchange (using DEAE cellulose 52 resin) and gel filtration chromatography (using Superdex 200 column). The purified enzyme had a retention time of 7 min in RP-HPLC. The enzyme was stable at a broad range of temperature (30-60°C) and pH (5.0-8.0) with a half-life of 58.72 min, V_{max} of 37.17 μM min/ mL and K_m of 0.657 mg/mL. Its activity was enhanced by Na^+, Ca^{2+}, Mg^{2+} ions and SDS surfactant. These properties make this enzyme a valuable candidate for industrial applications.

Keywords: Basidiomycetes, Central Composite Design (CCD), *Coriolus versicolor*, Edible mushrooms, Plackett-Burman Design (PBD), Polypore mushroom, *Polyporus versicolor*, Response Surface Methodology (RSM), Solid-state fermentation, *Trametes versicolor*, Turkey tail

A protease (peptidase or proteinase) is any enzyme that catalyses proteolysis and is involved in a wide range of physiological events, from basic protein digestion to carefully controlled cascades. They perform critical regulatory functions in all organisms such as conception, birth, digestion, development, maturity, ageing, and even death by managing the activation, synthesis, and turnover of proteins. Proteases are (physiologically) required for all living species. They are widespread and may be found in a wide range of environments. According to the MEROPS Database, proteases are divided into seven classes based on the active sites at which they operate during the hydrolysis of peptide bonds: metallo, aspartic, asparagine, cysteine, threonine, serine and glutamic[1]. Furthermore, they can also be categorized as acid, neutral and basic proteases based on the ideal pH at which they are active.

Although proteases are found in all creatures, microorganisms are the most abundant source of these enzymes. Proteases derived from plants, animals, and microorganisms account for over 60% of total enzyme sales. Because of their biochemical and physiological features, easy culture conditions, and simplicity of cell manipulation, microorganisms are the most frequent source of commercial enzymes. These enzymes are employed in food processing, medicines, leather processing, X-ray film industry silver recovery from X-ray films, industrial waste treatment, and as detergent additives[2]. Fermentative techniques are used to produce microbial proteases. The majority of commercial proteases are bacterial in origin and have undergone substantial research. Fungal enzymes are advantageous due to the simplicity with which cells may be removed during downstream processing. As a result, the potential use of fungal proteases is becoming more apparent[3].

The studies pertaining to proteases has been limited to several fungal genera including *Aspergillus*, *Mucor*, *Cephalosporium*, *Paecilomyces*, *Conidiobolus*, *Rhizopus*, *Fusarium*, *Tritirachium* and *Penicillium*. Comparatively, neutral proteases have been reported from genera such as *Aspergillus*, *Penicillium and Fusarium*. Proteases

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have been reported to be produced through submerged (SmF) and solid-state fermentations (SSF)[4-6]. Filamentous fungi are among the widely exploited species in SSF. The reason to this may be attributed to their potential to thrive on solid substrates and produce diverse extracellular enzymes[6]. The production and the activity of extracellular proteases isolated from fungi depends upon the composition of the media, mainly the carbon and the nitrogen sources; the physicochemical factors such as pH, incubation time, temperature and agitation[7].

Proteases from edible mushrooms (basidiomycetes) have been reported from *Agaricus bisporus*, *Armillariella mellea*, *Grifola frondosa*, *Flammulina velutipes*, *Peniciliium citrilopileatus*, *Pleurotus ostreatus* and *Pleurotus eryngii*[3,8,9]. *Trametes versicolor* (L.) Lloyd [Syn. *Coriolus versicolor* (L.) Quél.; *Polyporus versicolor* (L.) Fr.], commonly called as Turkey tail, is used in traditional medicine in China. The bioactive constituents of *T. versicolor* have reported tumor suppressing properties[10]. Presence of proteolytic agents has not been reported in *T. versicolor* till date. Therefore, in this study, we have made an attempt to identify protease enzyme in *T. versicolor* and report the production as well as optimization of the media for enzyme production using Response surface methodology (RSM).

Methods and Materials

Microbial Strain and growth

Trametes versicolor (MTCC 138) was procured from MTCC, Chandigarh. It was grown using Potato Dextrose Agar (PDA) comprising (g/L): potato 200, dextrose 20, and agar 20. The pH of the medium was adjusted to 6.5 using 1N HCl. The medium was inoculated using a loopful of cells and incubated at 30°C for seven days.

Bioassay using agar plate assay method

Tramets versicolor was grown on skim milk agar for protease assay by inoculating the culture on the skim milk agar plates and incubating at 30 °C for 96 h. The composition of the skim milk agar[11] was as follows (g/L): skim milk powder 28, agar 15, dextrose 1.0, yeast extract 2.5 and casein enzymatic hydrolysate 5. The pH of medium was adjusted to 6.5 using 1N HCl.

Production of protease

Solid-state fermentation was carried out for the production of protease by *T. versicolor*. For this, fungal spores were obtained from the revived strain of *T. versicolor* by extracting spores from the agar plate and suspending them in 10 mL of autoclaved distilled water. Wheat bran (15 g) was taken as the substrate and was added to a 250 mL Erlenmeyer flask, and 20 mL of minimal media was added. The pH was adjusted to 6.0 and autoclaved at 121°C for 15 min. Spore suspension (2×10^4 spores/mL), 2 mL was added to the flask and was incubated at 30°C for 7 d. The glucosamine content was estimated to determine the cell biomass produced during fermentation[12].

Extraction of crude enzyme

To extract the enzyme from the fermented solid-state substrate, it was mixed with distilled water (100 mL) containing 0.1% Tween 20 at 37°C on a rotary shaker for 2 h. The solution was filtered using a muslin cloth leading to the filtration of the fine particles along with the liquid content. The filtrate obtained was centrifuged at 8000×g for 15 min at 4°C. The clear supernatant obtained was preserved as crude extract at 4°C for the assay[13].

Protease assay

Folin and Ciocalteau method was used to estimate the activity of the protease enzyme. About 5 mL of 0.65% casein solution was added to each test tube and equilibrated in a water bath for 5 min at 37°C[14,15]. This was followed by addition of 2 mL enzyme containing crude extract to the test tube (trypsin as positive control and water as negative control). The protease activity and tyrosine liberation were measured and compared between the test samples. Further, 5 mL trichloroacetic acid, 5 mL sodium carbonate, and 1 mL Folin's reagent were added sequentially to the test tubes and incubated for 30 min at 37°C. The absorbance was taken at 660 nm. The amount of enzyme that liberated 1 µM of tyrosine from the substrate per min under the standard assay conditions was termed as one unit of enzyme. The activity of the enzyme was calculated in U/mL (µM of tyrosine released/min/mL).

Optimization of physicochemical parameters using Plackett-Burman (PB) and Central Composite (CC) Design

The production of the protease enzyme was statistically optimized by Plackett-Burman design (PBD)[16]. For carbon source (10%), wheat bran, pigeon pea bran, Bengal gram bran, rice bran and potato peel were taken while for the nitrogen source (2%), yeast extract, soybean meal, peptone, casein, ammonium sulphate, ammonium nitrate, sodium nitrate and casein and soybean (1:1) were added to the media.

Six variables were screened and through PBD each given a high (+) and low level (-) respectively, which were wheat bran (g), peptone (g), initial pH, moisture content (%v/w), temperature (°C), incubation time (h). The 12-run Plackett-Burman design providing significant variables were optimized using Response Surface Methodology (RSM) through 20-run Central Composite design (CCD) and the variables were represented at five different levels (+1.682, +1, 0, –1, –1.682). Following equation is employed to study the variable's effect:

$$E= (\sum M+ - \sum M-)/N$$

where E represent effect of the parameter; M+ and M, Protease inhibitor activity response; and N, number of trials. The second order polynomial equation was employed for the statistical optimization:

$$Y= \beta 0 + \sum \beta i\ Xi + \sum \beta ii\ Xi^{2} + \sum ij\ \beta ij\ XiXj$$

Extraction and purification of protease

The ammonium sulphate precipitation was employed to isolate the enzyme from crude extract[17]. The recovered pellets after the process were dialyzed against phosphate buffer 0.1 M, pH 7.0 (ratio 1:100) for 3 h, 6 h and overnight, respectively. The dialysate was recovered through centrifugation and purified using ion-exchange chromatography. The DEAE Cellulose-52 ion exchange column was equilibrated with phosphate buffer. The fractions were eluted out though application of 1M NaCl gradient using 0.2, 0.4, 0.6, 0.8, 1.0 M NaCl solution at 1 mL/min flow rate. The fraction (20 µL, 0.1 mg/mL) exhibiting maximum activity at 280 nm was subjected to gel filtration chromatography using HiLoad Superdex 200 (16/600 pg) column on AKTAPure 25M (Amershem Pharmacia Biotech, Sweden). Detection was performed at 280 nm. The fraction with maximum protease activity (%) was concentrated through centrifugation at 5000×g for 40 min at 4°C using Amicon Ultra-15 Centrifugal Filter Unit (Millipore, USA).

The purity of the concentrated gel filtration chromatography fraction was studied through reversed-phase HPLC. The fraction with maximum protease activity (20 µL, 0.1 mg/mL) was subjected to Sunfire C18 5.0 µm column and gradient elution was performed for 40 min[18]. The absorbance of the sample was detected through photodiode array (PDA) detector at 220 nm.

Characterization of protease

Effect of pH and Temperature on protease activity and stability

The effect of pH ranging from 4.0 to 10.5 on the protease activity was determined. To check the effect of pH on the stability, the enzyme was treated with buffer solution of respective pH for one hour after which the proteolytic activity was recorded.

Similarly, the effect of temperature from 30-80°C, at pH 7.5 was evaluated on enzyme activity using casein as substrate. The protease thermal stability was determined by 30 min incubation in sodium phosphate buffer. Furthermore, the supernatant was used to perform the protease activity assay. The experiment was done in triplicate.

Effect of metal ions, surfactant, and inhibitors

The protease at concentration of 1 mg/mL and metal ions (50 µL each), Ca^{2+}, Mo^{2+}, Cu^{2+}, Mg^{2+}, Na^{+}, Fe^{3+}, Mn^{2+}, Ba^{2+}, Zn^{2+}, and Al^{3+} at concentrations of 1 and 10 mM were incubated at 37°C for 60 min and residual protease activity was determined (%) in triplicates. The effect of different surfactants, Tween-20, Sodium dodecyl sulfate (SDS), Tween-80, and Triton X-100 was studied on the protease by incubating the enzyme (50 µL, 1 mg/mL) and the surfactant (50 µL) for 60 min at 37°C. The inhibitors, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), pepstatin, and E-64 (1 mM each) were pre-incubated for 1 h with Tris-HCl buffer. The control did not contain any inhibitor.

Study of kinetic parameters and Half-life of the purified protease

To study the kinetic parameters, the kinetic measurements were studied on enzyme-substrate reaction. It includes measurement of the enzyme-catalysed reactions rates at different enzyme and substrate concentrations using Michaelis–Menten kinetics.

To characterize an enzyme-catalysed reaction, K_m and V_{max} are required to be determined. The enzyme sample (1 mg/mL) was incubated with different concentrations of casein substrate and incubated at 30°C for 10 min. Following incubation, the reaction was stopped using 2.5 mL TCA and activity assay was carried out. The rate of catalysis (reaction velocity) was measured by determining V at various [S] values. Then plotting 1/V *vs*. 1/[S], a straight line was obtained called Double-Reciprocal Plot (also known as a Lineweaver-Burke plot). Michaelis – Menten constants K_m and V_{max} were determined from the slope and y-intercept.

For determination of half-life of the protease enzyme obtained from *T. versicolor*, 1 mL of the enzyme sample was taken in test tubes and incubated

at 50°C. Furthermore, at a time interval of 15 min 1 mL of substrate casein was added to the tube and incubated at 30°C for 10 min. The reaction was stopped using 2.5 mL TCA and activity assay was conducted.

Statistical analysis

The experiments performed in triplicates, and analyzed and expressed as mean±SD using GraphPad prism version 8.0.2 (263). The data were analyzed statistically by one way ANOVA with statistically significant *p* value of <0.05.

Results and Discussion

Bioassay and Biomass determination

Zone of inhibition was obtained in plates prepared of skim milk agar medium when the culture of *T. versicolor* and the crude extract were inoculated and poured into the assay wells. The protease produced led the degradation of the casein in the medium to give a clear zone indicating the production of protease. The bioassay using casein as a component of the assay plates confirmed the presence of a serine protease enzyme whose activity was calculated by using casein as substrate.

Glucosamine content determination for the biomass of the fungi led to the observation that *T. versicolor* entered the stationary phase after six days (144 h) of growth, and maximum activity of the protease enzyme was seen at 120 h (Fig. 1).

Optimization of physicochemical parameters using Plackett-Burman Design and Central Composite Design

Carbon and nitrogen sources used for optimization through PBD were chosen by screening different sources. For carbon source (10%), wheat bran gave the best results for protease production and for the nitrogen source (2%), peptone was found to be the most suitable for the growth of the mycelia and the production of the enzyme.

These two nutrient sources were used as variables for further screening of the physicochemical parameters. The Plackett-Burman Design (PBD) showed that three variables including temperature (^{o}C), incubation period (h) and wheat bran (g) had significant affect (P <0.05) (Table 1). The most significant of them was incubation time. The values were put in linear equation to determine the effect through ANOVA: Final equation in terms of coded factors:

Protease activity = +41.25+5.25* A+2.75* B+22.08* C+2.25* D-3.58* E-21.75* F

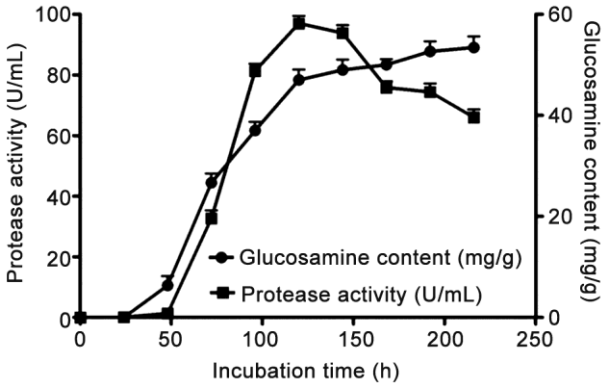

Fig. 1 — The graph representing the glucosamine content (mg/g) and protease activity (U/mL)

Table 1 — Analysis of variance for Plackett–Burman design

Source	Sum of squares	df	Mean Square	F Value	p-value Prob >F
Model	12165.17	6	2027.53	46.70	0.0003*
A-Wheat bran	330.75	1	330.75	7.62	0.0398
B-Peptone	90.75	1	90.75	2.09	0.2079
C-Incubation time	5852.08	1	5852.08	134.79	<0.0001
D-Initial pH	60.75	1	60.75	1.40	0.2900
E-Moisture content	154.08	1	154.08	3.55	0.1183
F-Temp.	5676.75	1	5676.75	130.75	<0.0001
Residual	217.08	5	43.42		
Cor Total	12382.25	11			

[*Significant. Level of significance P <0.05, S.D., 6.59; CV, 15.97%; R^2, 98.25%; Adjusted R^2, 96.14%; Predicted R^2, 89.90%]

where A,wheat bran (g); B, peptone (g); C, incubation time (h); D, initial pH; E, moisture content (%v/w); and F, temperature (°C).

The central composite design (CCD) was used to optimize the screened significant variables. The 20-run design response was summarized ANOVA (Table 2) in the polynomial equation as follows:

Final equation in terms of coded factors:

Protease activity = +96.80+1.89* A-12.40* B+22.61* C-0.25* AB+0.000* AC-10.25* BC-10.15* A^2-29.95* B^2-21.11* C^2

where A, wheat bran (g); B, temperature (°C); and C, incubation time (h).

The three-dimensional response surface plots (Fig. 2 A-C) of the three significant variables were generated by keeping one of them at zero level and studying the interaction between the other two variables. These plots gave the maximum production of the protease (102 U/mL) with the optimal growth factors. The model was validated using 10 g of wheat bran to produce the fungi at 30°C and incubated for 120 h (5 d). This increased the production of the enzyme from 78 U/mL (initial conditions provided for

T. versicolor growth) to 102 U/mL (statistically optimized media).

The significant variables: wheat bran (g), incubation time (h) showed a positive effect on the production of the enzyme while temperature had a negative effect. Protease enzyme is mostly produced in the early stationary phase, or late logarithmic phase and the growth is associated with mycelial growth[19,20]. This was validated through optimization, where it was seen that on the fifth day (120 h) of the fungal growth the production was maximum. In a previous study, *Aspergillus oryzae* MTCC 5341 showed maximum protease production at 120 h incubation time using wheat bran as substrate[21]. Moisture content has a substantial impact on the growth as solid state fermentation is defined by the amount of moisture retained by the media. In excess as well as in limitation, it eschews the growth of mycelia. In this case, it was kept at 50% v/w. Most SSFs have 40-70% moisture content depending upon the microorganism and the substrate[3]. The production of the protease enzyme was arrested above a temperature of 30°C. Temperature plays a crucial role in the growth of the fungus, and since the production of extracellular enzymes from white rot fungi is directly proportional to the mycelial expansion on the media, it gets affected by altering the temperature. Also, at higher temperature, the protease also tends to lose its catalytic activity due to breaking of the hydrogen bonds[22]. *Aspergillus sp*. has reported production of protease enzyme at 28°C[23]. Similarly, *Lentinus sajor-caju* (formerly called as *Pleurotus sajor-caju*) gave maximum production of the protease at 30°C.

Peptone (2%) was found to be the suitable nitrogen source for *T. versicolor* growth and enzyme production. Several reports have substantiated the use of peptone as one of the best nitrogen source for protease production from different fungi[24,25]. The carbon substrate for the production used here was an inexpensive agro-industrial raw material, wheat bran, which is readily available and its feasibility in usage as an anchorage for the mycelia of the fungus. It showed higher protease production owing to higher fungal biomass generation as compared to other raw materials. Solid state fermentation using wheat bran as the primary substrate has been extensively used for extracellular enzyme production from other fungal sources such as *Aspergillus niger*, *A. oryzae*, *Penicillium* sp., *Rhizopus oryzae* and *Thermoascus aurantiacus*[3]. The statistical optimization not only validated the production but also enhanced the production in statistically optimized media. It was observed that production of the protease enzyme

Table 2 — Analysis of variance for central composite design

Source	Sum of squares	df	Mean square	F value	p-value Prob >F
Model	28138.38	9	3126.49	122.31	<0.0001*
A-Wheat bran	48.64	1	48.64	1.90	0.1978
B-Temp.	2101.45	1	2101.45	82.21	<0.0001
C-Incubation time	6981.07	1	6981.07	273.10	<0.0001
AB	0.50	1	0.50	0.020	0.8915
AC	0.000	1	0.000	0.000	1.0000
BC	840.50	1	840.50	32.88	0.0002
A^2	1485.20	1	1485.20	58.10	<0.0001
B^2	12927.60	1	12927.60	505.73	<0.0001
C^2	6423.29	1	6423.29	251.28	<0.0001
Residual	255.62	10	25.56		
Lack of Fit	196.79	5	39.36	3.34	0.1056**
Pure Error	58.83	5	11.77		
Cor Total	28394.00	19			

[*Significant, ** not significant. Level of significance P <0.05, S.D., 5.06; CV, 9.19%; R^2, 99.10%; Adjusted R^2, 98.29%; Predicted R^2, 94.44%]

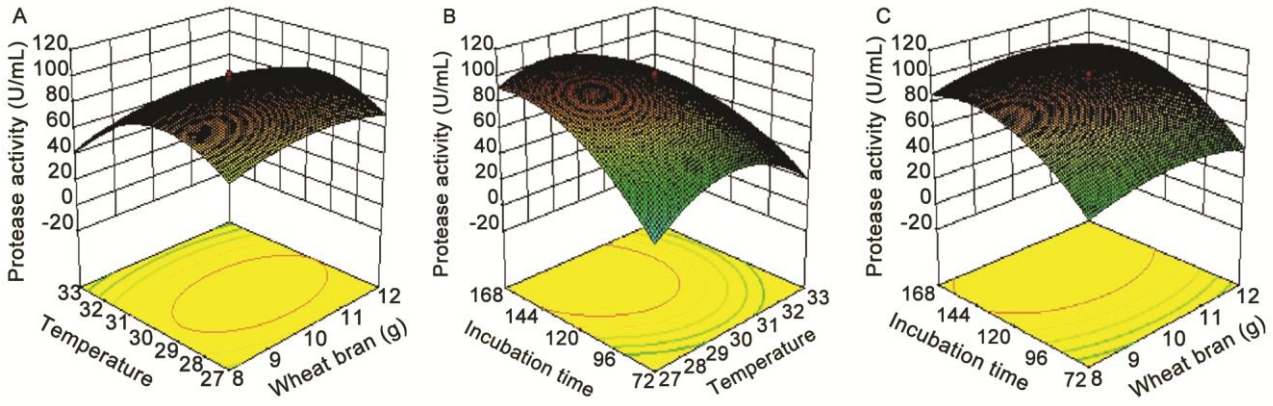

Fig. 2 — Three-dimensional surface response plots to study the effect of (A) wheat bran and temperature; (B) temperature and incubation time; and (C) wheat bran and incubation time, on the protease production

increased by 30.76% in optimized media formulation in comparison to control.

Purification of protease

The protease enzyme fraction of 60-80% ammonium sulphate exhibited highest protease activity. The highest protease activity fraction (Fig. 3A) was further purified using Superdex 200 (16/600 pg) column on AKTA PURE25 FPLC system. The unbound proteins eluted with sodium phosphate buffer and the linear gradient of 1 M NaCl eluted protease enzyme at 0.4 M. In gel filtration chromatography, the purified enzyme eluted at 102 min (Fig. 3B). Reversed Phase-HPLC was then performed to determine the purity and characteristic of the enzyme. The peak at 7 min confirmed the purity of the protease enzyme (Fig. 3C).

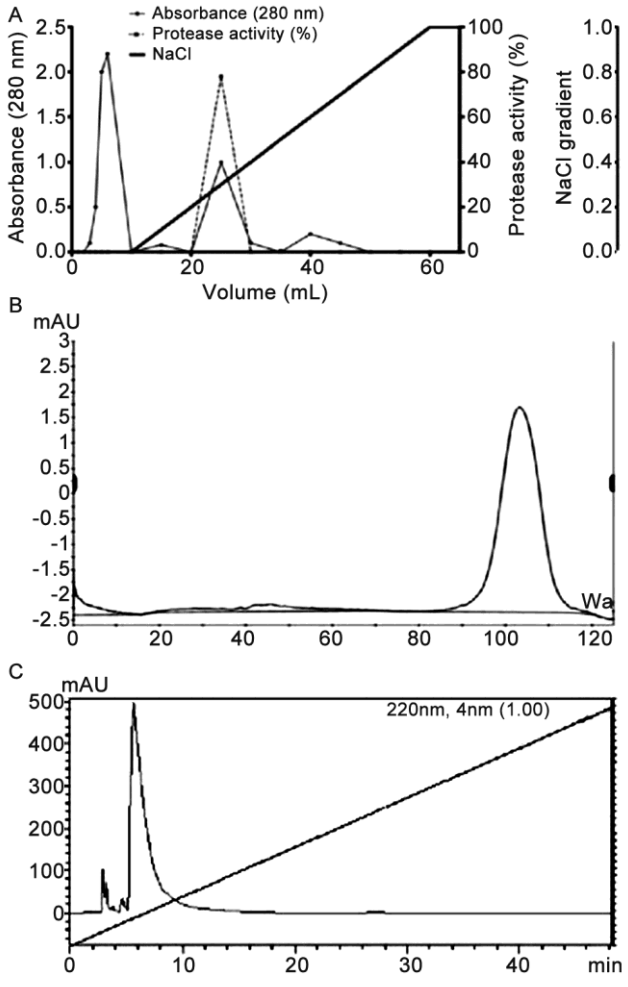

Fig. 3 — (A) DEAE cellulose column chromatography elution profile of the sample showing the gradient elution; (B) Gel filtration chromatography with Superdex 200; (C) Reversed phase HPLC profile of the protease enzyme isolated from *T. versicolor* after gel filtration chromatography

In the precipitation stage, the protease activity was observed in 60-80% ammonium sulphate saturation indicating the presence of more hydrophilic residues in the protease enzyme. More amount of salt is required to overcome the hydrophilic association between surrounding water molecules and protein if more hydrophilic entities are present in protein. Higher percentage of ammonium sulphate confirmed the same. The protease enzyme was eluted in ion exchange chromatography through DEAE cellulose 52 column at 0.4 M NaCl concentration during the linear NaCl gradient. The eluted enzyme was passed through Sephadex 200 column. The late elution at 102 min confirmed the low molecular weight of the protease enzyme establishing the similarity with other fungal proteases, as fungal proteases are generally of low molecular weight[26]. RP-HPLC of the enzyme fraction from gel filtration column confimed the purity with a peak at 7 min. The early elution of the enzyme from the column reiterated the observation of precpitation stage that the protease enzyme had more hydrophilic residues in proportion to the hydrophobic residues.

Characterization of protease

Effect of pH and temperature on protease activity and stability

The maximum activity of protease obtained from *T. versicolor* was observed at pH 7.0 and was stable over a wide pH range of 5.0 to 8.0. Maximum activity of the enzyme was observed at 50°C for protease and was stable from 30-60°C. The results indicated that the produced protease was a neutral and thermophilic enzyme. Similar results were obtained for protease produced from other fungal sources. A study on *Graphium putredinis* and *Trichoderma harzianum* fungi reported similar observations where the proteases isolated from these fungi exhibited maximum protease activity at pH 7.0 and temperature 50°C[27]. Proteases isolated from the basidiomycete *Pleurotus* sp. have an optimum pH and temperature range substantiating results of the pH and temperature from *T. versicolor*[26]. Protease from *T. versicolor* was stable at a range of pH 5.0-8.0 and high temperature 30-60°C. This is in corroboration with the study on the proteases from *Grifola frandosa* (ProGF) that showed highest activity at temperature 55°C and pH 7.0, and were stable within 4.5-8.5 pH range and 50-70°C temperature range using casein as substrate[28]. Proteases from basidiomycetes are known for their broad pH and temperature working range, most of which exhibit thermostablity[23,26,28-31].

Effect of metal ions, surfactants and inhibitors on protease activity

Ca^{2+}, Mg^{2+}, and Na^{+} (1 and 10 mM) enhanced the activity o the protease enzyme while Zn^{2+}, Cu^{2+}, Fe^{3+}, Ba^{2+}, Mo^{2+} and Al^{3+} decreased the activity of the enzyme. The activity was enhanced in the presence of 1 mM and 10 mM of Ca^{2+}, Mg^{2+} and Na^{+} ions to 105 and 111%, 102.333 & 109.67% and 110 & 114.66%, respectively comparised to control (100%) while all other metal ions significantly inhibited the activity of the enzyme (Fig. 4).

The protease from *T. versicolor* is a serine protease and this feature can be attributed to the increased activity in the presence of Ca^{2+} ions. Serine proteases have two calcium binding sites and the presence of Ca^{2+} ions aids in the maintenance and enhancement in the stability of the three dimensional conformation of the protein. Divalent metal ions are reported to stabilize the tertiary and quaternary protein structures[24]. Serine proteases from basidiomycetes such as *Lentinus sajor-caju* and *Flammulina velutipes* have reported enhancement in activity in presence of Ca^{2+}[30,31]. The activity of the protease in the present study also increased in presence of Na^{+} and Mg^{2+} ions. These metal ions have previously been reported to enhance the catalytic functioning of fungal serine proteases[28,30-33].

Protease activity increased in the presence of anionic surfactant SDS (1%) to 122% in comparison to control (100%) whereas the non-ionic surfactants Tween-80, Tween-20, and even Triton X-100 had a slight detrimental effect on the activity by reducing it to 87, 83 and 93%, respectively. The increase in the activity of the protease enzyme in the presence of SDS (1%) signifies that SDS functioned as a stabilizer to the three dimensional structure of the enzyme and rearranged it to interact with the hydrophilic amino acids of the polypeptide chain, enhancing the solubility of the enzyme in solution. On the contrary, the non-ionic surfactants may have exploited the structure in such a way that the hydrophobic amino acids were exposed and interacted with them, reducing the activity and stability of the enzyme[34]. Similar trend in increase in activity in presence of SDS has been previously reported in bacterial proteases from *Bacillus* sp.[35], and in fungal protease from *Aspergillus foetidus*[24].

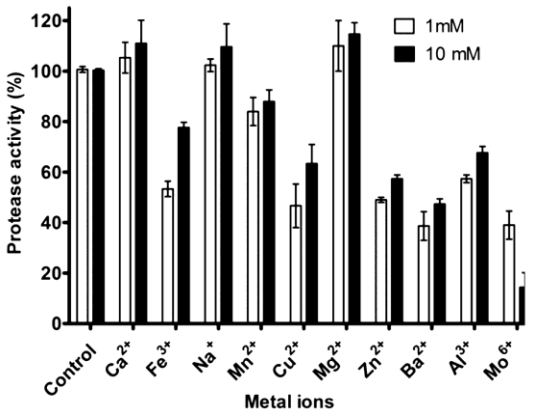

Fig. 4 — Effect of metal ions on the protease activity.

The enzyme was completely inhibited by PMSF (a serine protease inhibitor). Other inhibitors: EDTA, Pepstatin, and E-64 had no detrimental effect on the activity of the enzyme. The result indicated that the protease enzyme isolated from *T. versicolor* was a serine protease. The serine proteases are found mostly in all fungi, accounting for 77% of fungal proteases, and are generally extracellular. The molecular weight of the fungal proteases ranges from 18-35 kDa and these are mostly alkaline and thermostable[3,26,36].

Kinetic parameters K_m and V_{max} and half-life of the protease

The values of Michaelis-Menten constants, V_{max} and K_m were 37.17 μM/min/ mL and 0.657 mg/ mL respectively, for the protease enzyme produced from *T. versicolor* (Fig. 5A). The half-life of the protease enzyme was 58.72 min at 50°C (Fig. 5B). The kinetic parameter values obtained indicated the protease's dynamic binding to the substrate. Previously, the purified proteases SPPS from *Lentinus sajor-caju* CTM10057, SPTC from *Trametes cingulata*,

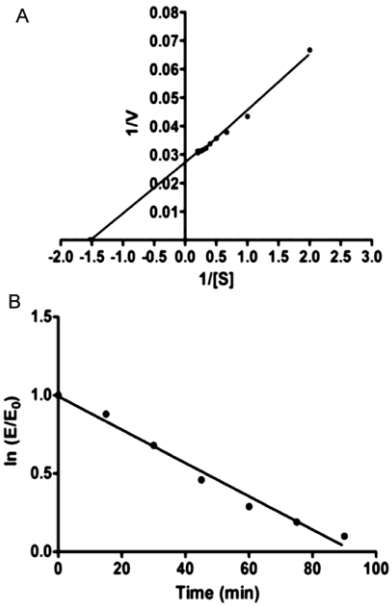

Fig. 5 — Kinetic parameters of the protease enzyme from *T. versicolor*: (A) Lineweaver Burk Plot to determine K_m and V_{max} of the protease produced from *T. versicolor*; and (B) Determination of half-life of protease enzyme at 50°C

CTM10101, Thermolysin type X from *Geobacillus stearothermophillus*, and Flavourzyme 500 L from *Aspergillus oryzae* reported K_m values of 0.275, 0.475, 0.613 and 0.546 mg/mL, respectively[31]. These values suggest that the protease in the present study exhibited similar affinity towards casein as did the other fungal proteases.

Conclusion

The present study utilized low cost raw materials for the production of a serine protease from wood-rot fungi *Coriolus versicolor*. The protease production media was optimized statistically through Response Surface Methodology, thus aiding its enhanced output (102 U/mL) by providing the precise formulation. The enzyme appeared to be a low weight molecule exhibiting a broad range pH and temperature activity (30-60°C). The results of the present study can be substantiated with further research on the amino acid sequence deciphering its structure. Being a neutral and thermostable protease, this enzyme may be utilized in detergent formulations, food and therapeutics.

Conflict of interest

Authors declare no competing interests.

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