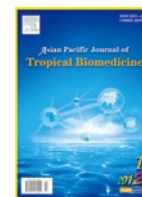




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Document heading

# A protease from the medicinal mushroom *Pleurotus sajor-caju*; production, purification and partial characterization

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## ABSTRACT

**Objective:** To produce protease using solid state fermentation from *Pleurotus sajor-caju*. **Methods:** Optimization of saccharification conditions like time, pH, temperature, inoculum size, different carbon sources and its concentration, various nitrogen sources and its concentrations were studied. **Results:** The results showed that the optimum conditions for maximum protease production under SSF (Solid State Fermentation) by *Pleurotus sajor-caju* was found to be on the fourth day of incubation, pH, 7; temperature, 30°C, 3%; corn as substituted carbon source; 0.8% ammonium nitrate as nitrogen source concentration. Protease was partially purified by ammonium sulphate precipitation (70% saturation) to 2.72 fold with recovery of 53% characterized for few properties. Homogeneous preparation of protease from the culture filtrate of the fungus has been achieved using ammonium sulphate precipitation, anion exchange chromatography on DEAE cellulose. The purified enzyme preparation gave a single protein band in SDS-PAGE analysis indicating a molecular weight of 48 kDa. The purified enzyme exhibited its optimal activity at 60 °C and at pH 8. Metal ions (Ca<sup>2+</sup> and Na<sup>2+</sup>) caused moderate reduction in enzyme activity. **Conclusions:** Considering the importance and application of the proteases, this study provides better understanding of condition for the activity of protease by fungal culture.

## 1. Introduction

Protease constitutes a large and complex group of enzyme which plays an important nutritional and regulatory role in nature. Proteases are (physiologically) necessary for living organisms; they are ubiquitous, found in a wide diversity of sources. Protease is the most important industrial enzyme of interest accounting for about 60% of the total enzyme market in the world is 40% of the total worldwide enzyme sale[1,2]. Proteases are essential constituents of all forms of life on earth including prokaryotes, fungi, plants and animals. These are highly exploited enzymes in food, leather, detergent [3], pharmaceutical, diagnostics, waste management and silver recovery[4–7].

Proteases are extracellular enzymes that can be

produced by both submerged fermentation and solid state fermentation. Solid state fermentation (SSF) is especially suited to growth of fungi because of their lower moisture requirements compared with bacteria[8,9]. Economically, SSF offers many advantages, including superior volumetric productivity, use of simpler machinery, use of inexpensive substrates, simpler downstream processing and lower energy requirements when compared with submerged fermentation[6]. Several species of strains including fungi (*Aspergillus flavus*, *Aspergillus melleu*, *Aspergillus niger*, *Chrysosporium keratinophilum*, *Fusarium graminearum*, *Penicillium griseofulvin*, *Scedosporium apiosermum*) and bacteria (*Bacillus licheniformis*, *Bacillus firmus*, *Bacillus alcalophilus*, *Bacillus amyloliquefaciens*, *Bacillus proteolyticus*, *Bacillus subtilis*, *Bacillus thuringiensis*) are reported to produce proteases[10]. In the present study an enzyme protease was isolated from the medicinal mushroom *Pleurotus sajor-caju*, partially purified and characterized. This study was started as a survey of new species to learn their capacity in the production of protease enzyme under solid state fermentation.

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## 2. Materials and methods

### 2.1. Selection of organism

The organisms present in the pure culture were identified using lactophenol cotton blue mounting method, which was especially used for the identification of fungi. The mushroom used throughout the experiment was *Pleurotus sajor-caju* collected from Tamil Nadu Agriculture University, Coimbatore. The culture was maintained in Rose Bengal medium.

### 2.2. Fermentation condition

SSF was carried out in 250 mL conical flask contains 10 g of substrate with 10 ml of salt solution (g/L). KNO<sub>3</sub> 2.0, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5, K<sub>2</sub>HPO<sub>4</sub> 1.0, ZnSO<sub>4</sub>·7H<sub>2</sub>O 0.437, FeSO<sub>4</sub>·7H<sub>2</sub>O 1.116, MnSO<sub>4</sub>·7H<sub>2</sub>O 0.203, pH 7.0 and it was autoclaved at 121°C for 30 min. After sterilization, the flasks were inoculated with 1.0 mL of spore solution (10<sup>6</sup> spores/mL) and incubated at 30 °C for eight days in an incubator shaker at 125 rpm. At the end of fermentation, cultures were extracted with 100 mL of distilled water by shaking for 2 hours. The filtrate obtained was centrifuged at 10 000 rpm for 10 min at room temperature. The supernatant was used as crude enzyme extract.

### 2.3. Protease assay

Protease activity was determined according to the modified Anson's method. 1.0 mL of the culture broth was taken in a 100 ml flask and 1.0 mL of pH 7.0 phosphate buffer added to it. One mL of the substrate (2% Hammersten's casein pH 7.0) was added to the buffer-enzyme solution and incubated at 37 °C for 10 minutes in a water bath. At the end of 10 minutes, 10.0 mL of 5N TCA (trichloroacetic acid) was added to stop the reaction. The precipitated casein was then filtered off and 5.0 mL of the filtrate were taken in a test tube. To this 10.0 mL of 0.5 N NaOH and 3.0 mL of the Folin ciocalteu reagent (one ml diluted with 2 mL of distilled water) were added. Final readings were taken in a spectrophotometer at 750 nm. Blanks of the samples were prepared by adding TCA before the addition of substrate.

### 2.4. Optimization of process parameters

Various process parameters affecting enzyme production during SSF were optimized. The strategy was to optimize each parameter independently of the others and subsequently optimal conditions were employed in all experiments. The best solid substrate was selected for optimum production of protease and the suitable solid substrate was used in subsequent experiments. The tested process parameters in this study were inoculum concentration (1%–5%, v/w), incubation time (24, 48, 72, 96, 120, 144, 168, 192 h), incubation temperature (20–60 °C), initial pH (3–8), supplementary

carbon sources (wheat bran, rice bran, green gram, corn flour and ragi) and its concentration (1%–5%), nitrogen sources (ammonium nitrate, ammonium sulphate, potassium nitrate, potassium chloride and sodium nitrate) and its concentration (1%–5%). On the basis of experimental data corn flour was found to be the best solid substrate in solid state fermentation process.

### 2.5. Purification and characterization of Protease

Crude extract was precipitated by 70% saturation with ammonium sulphate and then dialyzed against 50mM phosphate buffer (pH 7.0) for 24 hours at 40 °C. The filtrate was loaded onto a DEAE-Cellulose chromatographic column equilibrated with phosphate buffer, 50 mM, pH 7.0. The enzyme was eluted with a linear salt concentration gradient (NaCl, 0–0.4 M) in the same buffer and 3.0 mL fractions were collected at a flow rate of 20 mL per hour.

SDS-PAGE electrophoresis was carried out and molecular weight was determined. The protein content was estimated by the method of Lowry *et al.* The kinetic parameter of the purified protease enzyme was determined and the optimum pH 3.0 –8.0 [The pH was adjusted using, the following buffers: 50 mM sodium citrate (pH 3.0–6.0) and 50 mM sodium phosphate (pH 7.0 & 8.0)], temperature (30–70 °C), substrate concentration (1%–5%) and metal ions (Zn<sup>2+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup>, Na<sup>+</sup> and Cu<sup>2+</sup>) on the activity of the enzyme was also assayed. All experiments were conducted in triplicates and their mean values represented.

## 3. Results

Different agricultural byproducts such as wheat bran, rice bran, green gram, ragi and corn flour were tested for the production of enzyme (Figure 1). Of all the substrates tested, corn flour was found to be the best substrate for the production of protease. The other substrates gave comparatively less production of protease.

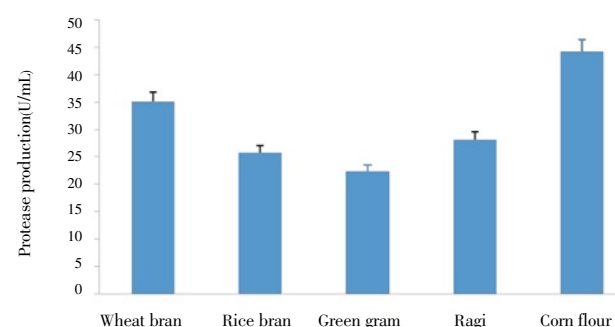


Figure 1. Effect of various substrates on protease production

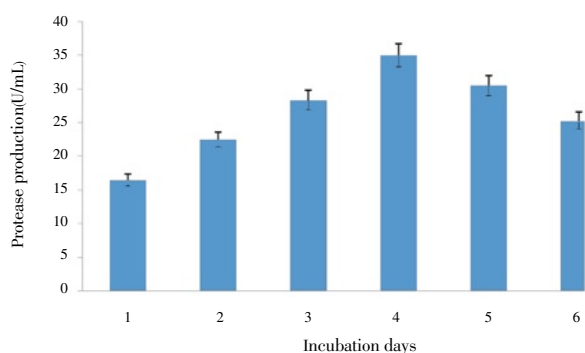
Results are mean of three independent determinations. Bars correspond to standard deviation.

The solid state fermentation medium was inoculated with

**Table 1**  
Purification and recovery of protease from *Pleurotus sajor-caju*.

Steps	Protease production (U)	Total protein (mg)	Specific production(U/mg)	Purification (fold)	Recovery (%)
Crude extract	130.00	31	4.19	1.00	100.00
70% Ammonium sulphate precipitation	114.00	26	4.38	1.04	87.69
Dialysis	95.34	21	4.54	1.08	73.33
DEAE Cellulose Column Chromatography	68.50	6.0	11.41	2.72	52.69

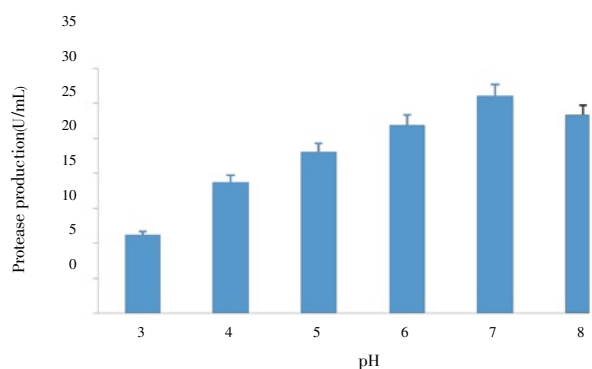
the fungal strain and incubated for various time intervals (1–6 days). Figure 2 shows the effect of incubation time on protease production. *Pleurotus sajor-caju* produced highest amount of enzyme on the fourth day of incubation.



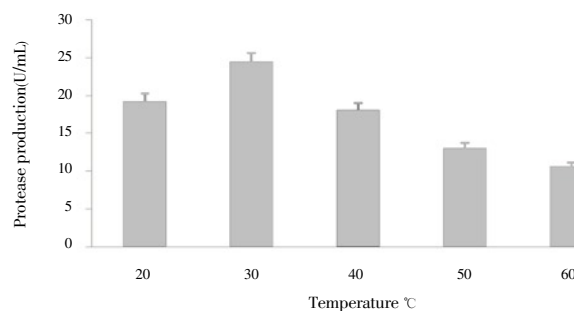
**Figure 2.** Effect of incubation dayson protease production

Productivity of the enzyme by culture is very much dependant on pH of the fermentation medium. Therefore, the effect of pH (3.0 – 8.0) was studied for the production of protease by *Pleurotus sajor-caju*. The maximum enzyme production occurred at pH 7.0 (Figure 3).

Incubation temperature of the fermentation medium is a critical factor has insightful influence on metabolic activities of microorganisms. The effect of different incubation temperature (20–70 °C) on the protease production was investigated. The production of enzyme was maximal in flasks incubated at 30 °C (Figure 4). As the temperature increased, there was a gradual decrease in the enzyme production.

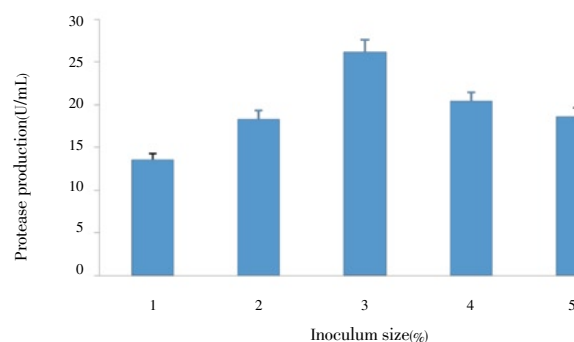


**Figure 3.** Effect of pH on protease production.



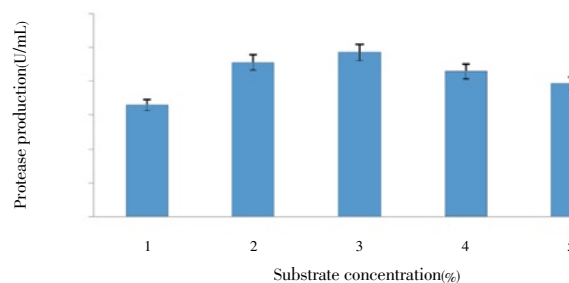
**Figure 4.** Effect of temperature on protease production.

Size of inoculum is an important biological factor in production of enzyme. Maximum enzyme production (Figure 5) was obtained when SSF medium was inoculated with 3.0 ml of inoculum.



**Figure 5.** Effect of inoculum size on protease production.

Protease production in fermentation medium was found to be maximal when 4.0% of wheat bran was used (Figure 6). Further increase in amount of wheat bran resulted in decrease in the production of enzyme.



**Figure 6.** Effect of substituted substrate concentration(cornflour) on protease production.

The higher C/N ratio of the medium composition results in pH decrease and lower C/N ratio of the medium composition results in pH increase. Consequently, increasing nitrogen concentration is another way to prevent pH decreasing during cell cultivation. The nitrogen source used in the production medium is one of the major factors affecting enzyme production and level. All the nitrogen sources (ammonium nitrate, ammonium sulphate, potassium nitrate, potassium chloride and sodium nitrate) used in the study (Figure 7) favored the synthesis of protease complex. In that, ammonium nitrate was found to be the most suitable nitrogen source for protease production. The influence of various concentrations of ammonium nitrate was investigated (Figure 8). In that, 3% ammonium nitrate was found to be best in protease production.

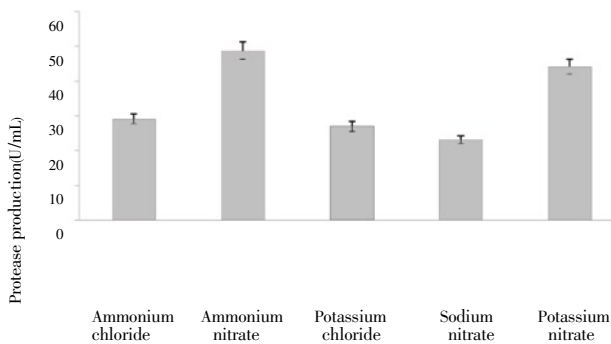


Figure 7. Effect of nitrogen source on protease production.

The results of purification steps of protease of *Pleurotus sajor-caju* are presented in Table 1. The initial step of purification was the precipitation of proteins with 70% ammonium sulfate, which gave 1.08 folds with yield 87.79% of the original activity. The second step of purification protease was made by chromatography on a DEAE cellulose column. The protease activity was located in one peak and reached 2.72 fold of purification.

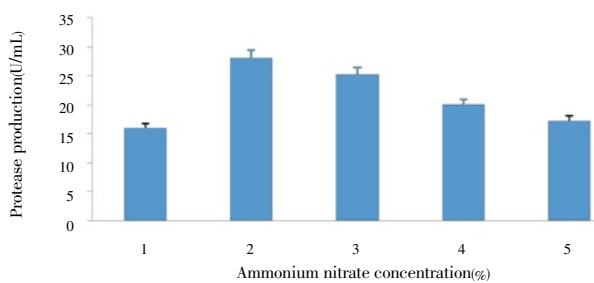


Figure 8. Effect of ammonium nitrate concentration on protease production

The molecular weight of the purified enzyme analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue showed a single band of approximately 48 kDa (Fig. 9) corresponding to the protease enzyme based on its high

activity on corn flour substrate.

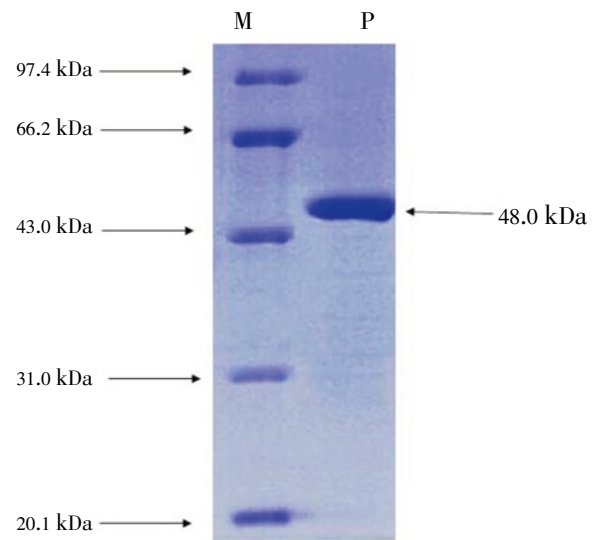


Figure 9. SDS-PAGE analysis of protease purified from *Pleurotus sajor-caju*.

Activity assay of protease was done in reaction mixture at varying pH by using appropriate buffers. It was found that enzyme has got activity over a broad range of pH (Figure 9). Maximum activity was expressed at pH 8 in case of corn flour as substrate.

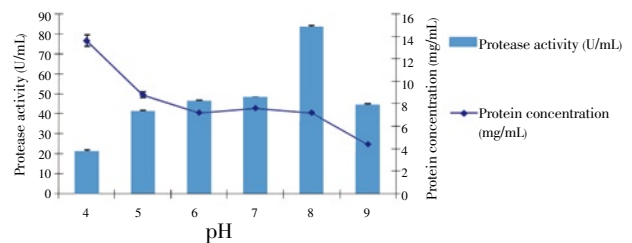


Figure 10. Effect of pH on protease activity.

For estimation of optimum temperature of enzyme, the enzyme activity was determined by carrying out the assay at several temperatures between 30 and 70°C. The optimum temperature was observed around 60°C.

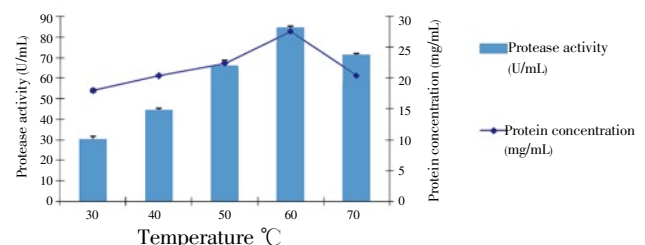
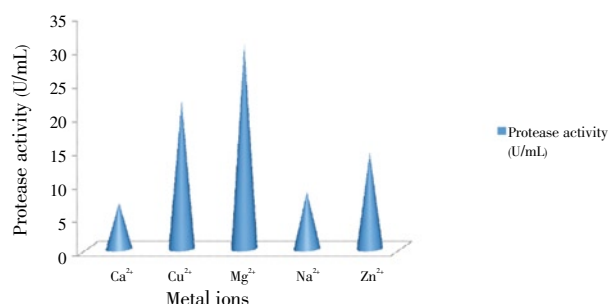


Figure 11. Effect of temperature on protease activity.

The effects of various ions on activity of the protease of *Pleurotus sajor-caju* is shown in Figure 12. Significant

inactivation of the protease of *Pleurotus sajor–caju* was observed with  $\text{Na}^{2+}$  but  $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Zn}^{2+}$  enhanced the enzyme activity.



**Figure 12.** Effect of metal ions on protease activity.

#### 4. Discussion

Enzyme production by micro organisms is greatly influenced by media components, especially carbon and nitrogen sources, and physical factors such as temperature, pH, incubation time and inoculum density. It is important to produce the enzyme in inexpensive and optimized media on large scale for the process to be commercially viable, hence the influence of various physico–chemical parameters such as incubation periods, inoculum size, temperature, pH, carbon, and nitrogen sources were studied. Agricultural byproducts rich in cellulosic biomass can be exploited as cheap raw material for the industrially important enzymes and chemicals<sup>[11]</sup>.

Microbial proteases have a number of commercial applications in industries like food, leather, meat processing and cheese making. A major commercial use is the addition of microbial proteases to domestic detergents for the digestion of pertinacious stains of fabrics<sup>[12]</sup>. It has been reported that the production of extracellular proteases by different microorganisms can be strongly influenced by the culture conditions. So it becomes necessary to understand the nature of proteases and their catalytic potentiality under different conditions.

There are general reports showing that different carbon sources have influences on extra cellular enzyme production by various strains. Among the various substrates (wheat bran, rice bran, green gram, corn flour and ragi) tested corn flour was found to be the most effective substrate for the production of protease with the concentration of 3%. Further increase in this carbon source adversely affected protease production in *Pleurotus sajor–caju* under SSF environment. It might be due to the fact that increased level of substrates decreases the aeration and porosity of the medium, which were very essential for the proper growth of the organism. Our results were supported by Radha *et al*<sup>[13]</sup> who reported that rice flour at 1% enhanced the protease production from 2.918 U/mL to 3.167 U/mL with maximum biomass growth of 86.4 mg/mL followed by corn flour with an activity of 3.068 U/mL.

In our study, the maximum enzyme activity was observed

at fourth day of incubation. This is in line with the findings of Sindhu *et al*<sup>[14]</sup> who reported that the enzyme could be harvested at about 96 hours of fermentation when the activity is highest. It might be due to the fact, that organism entered in the stationary phase of growth. Further increase in the incubation period led to a decrease in the production of protease by *Pleurotus sajor–caju*.

Several investigators have reported that the pH is an important factor which affects the growth and enzyme production during solid–state fermentation<sup>[15, 16]</sup>. Therefore, the effect of initial pH (4.0 – 9.0) was studied for the production of protease by *Pleurotus sajor–caju*. There was gradual increase in the amount of protease synthesis from pH 4.0 to 6.0 and maximum production of enzyme was observed at pH 7.0 i.e. 31.2 U/mL. However, pH of the fermentation medium beyond 7.0 resulted in a marked decrease in the production of protease. These data are in conformity to the findings of Vamsi *et al*<sup>[17]</sup> who have also made similar results with *Penicillium* sp. who noted the highest yield of protease at pH 6.6 of fermentation medium. Our results were contradictory to Kuberan *et al*<sup>[18]</sup> who reported that the maximum protease production was at pH 8. It is likely that changes in pH cause denaturation of enzyme resulting in the loss of catalytic activity. Therefore, each enzyme has specific pH optima for its activity.

Protease production by microbial strains strongly depends on the extra–cellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production<sup>[12]</sup>. *Pleurotus sajor–caju* showed maximal protease production at pH – 7. Our results were supported by Chutmanop *et al*<sup>[9]</sup> who reported that the protease production was maximum at pH of 7.5 for *Aspergillus oryzae* under solid state fermentation.

Medium temperature plays an important role in the protease production. Optimum temperature for the production of protease by *Pleurotus sajor–caju* was 30 °C. Identical observations were earlier recorded in different species of *Penicillium* including *Penicillium citrinum*, *Penicillium perpurogenum*, *Penicillium funiculosum* and *Penicillium griseoroseum*<sup>[12, 19]</sup>. Our results were also more or less same to Sindhu *et al*<sup>[14]</sup> who reported that the maximum protease production occurred at 35 °C for *Penicillium godlewskii* SBSS. The enzyme is denatured by losing its catalytic properties at high temperature due to stretching and breaking of weak hydrogen bonds within enzyme structure<sup>[20]</sup>.

At lower inoculums levels the yield was very low. The decrease seen with large inoculums size could be due to the shortage of the nutrients available for the large biomass and faster growth of the culture<sup>[21]</sup>. In our study, the maximum protease synthesis was noticed with 3% inoculum size whereas at higher concentration there was a decrease, it might be due to clumping of cells which could have reduced sugar and oxygen uptake rate and also enzyme release which

was almost supported by Bhatiya<sup>[22]</sup>.

All the nitrogen sources used in the study favored the synthesis of protease complex but there was a slight difference in the yield of enzyme. Ammonium nitrate with 2% was found to be most suitable nitrogen source for the production of protease production. Radha *et al.*<sup>[13]</sup> used various organic and inorganic nitrogen sources in their studies and who reported that the maximum enzyme activity was obtained with potassium nitrate followed by ammonium nitrate, when used as nitrogen sources.

The purification of crude enzyme through DEAE cellulose column chromatography gave 2.72 folds increase in purity with 53% recovery of protease from *Pleurotus sajor-caju* where as Almas *et al.*<sup>[23]</sup> reported that the protease was purified up to 11 folds for *Bacillus SAL1*. Fractions from DEAE–Cellulose column which showed the highest activity were pooled and subjected to SDS–PAGE for determination of molecular weight of the protein. Purified enzyme preparation showed only one band corresponding to molecular weight of approximately 48 kDa. Our results are more or less similar to that of Akel *et al.*<sup>[24]</sup> who reported that the purified protease enzyme revealed a molecular mass of 49 kDa.

The optimum pH values for protease in this study were 8.0 for *Pleurotus sajor-caju*. The optimal pH for purified protease produced *Bacillus subtilis* was 8.025. The protease enzyme from the mushroom *Termitomyces albuminosus* had an optimum pH of around 10<sup>[26]</sup>. Moreover, Lee<sup>[27]</sup> reported that, the optimum pH of purified protease was pH 8.

Since temperature influences protein denaturation, enzyme inhibition and cell growth, it plays a significant role in development of the biological process<sup>16</sup>. Jinka *et al.*<sup>[28]</sup> reported that cysteine protease secreted by germinating cotyledons of horse gram of industrial significance at optimum temperature of 40 °C. Similarly, Geethanjali and Subhsh<sup>[29]</sup> also reported that the optimum temperature for protease from *Bacillus subtilis* was found to be 40 °C.

The metal ions in media are an important factor that affects enzyme production due to act as inducers. The effects of some metal ions on protease activities were investigated. It has been found that Na<sup>2+</sup> but Ca<sup>2+</sup> inhibitor of protease enzyme and Cu<sup>2+</sup>, Mg<sup>2+</sup> and Zn<sup>2+</sup> was found to be the activator of protease enzyme. In contrast, Sevinc and Demirkan<sup>[30]</sup>; Kalaiarasi and Sunitha<sup>[31]</sup> reported that Ca<sup>2+</sup> and Mg<sup>2+</sup> ions were effected on protease production.

In the development of an optimized process for commercial production of microbial products, selecting a suitable low-cost nutrient medium and establishing the most favorable fermentation conditions are the two most important components<sup>[32]</sup>. In fermentation studies, the selection of a microbial strain having high capacity to produce a product is also very important. In this study, these aspects were considered for improvement. The fungal isolate having high capacity to produce protease was isolated. A cheap substrate

was explored and culture conditions were optimized. As seen from the table, the optimization of nutritional and environmental conditions significantly improved protease production. The present study showed that an agricultural waste material corn flour could be effectively utilized as substrate in solid–state fermentation for the production of protease which is extensively used in food industries. Therefore, it is very important for industrial, environmental and food microbiology.

### Conflict of interest statement

We declare that we have no conflict of interest.

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