

# Medium Formulation and its optimization for increased protease production by *Penicillium* sp. LCJ228 and its potential in blood stain removal

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## ABSTRACT

The production of protease by *Penicillium* sp. LCJ228 was optimized under submerged fermentation. Nutritional and physical factors that influence protease production were optimized by one factor at a time (OFAT) method in order to achieve high yield of protease. Maximum protease production was obtained on the 4<sup>th</sup> day in a liquid medium containing glucose (15 g/L), yeast extract (15 g/L), black gram husk (10 g/L) with an initial pH of 10 and an inoculum size of 2 g/L of fungal mycelium. About 2.4 fold increase in protease production was observed in the optimized medium. The maximally yielded crude protease was then precipitated and characterized by SDS-PAGE and native-PAGE. The molecular weight of the ammonium sulphate precipitated protease was ~66 to 70 kDa and native-PAGE showed three isoenzymes. The crude and precipitated protease had the ability to completely remove blood stains on cotton fabric indicating its potential to be used as a stain remover in detergents.

## 1. INTRODUCTION

Proteases are the most significant group of the industrial enzymes that execute a wide variety of functions and are commercially used for various industrial purposes [1, 2]. They have widespread applications in detergents, food, pharmaceuticals and leather industries [3, 4]. Proteases account for about 60% of the total enzyme market in the worldwide enzyme sale [5]. Increasing demand of proteases had lead researchers to search promising sources of proteases [6, 7]. Proteases are generally synthesized and produced by numerous microorganisms which include bacteria, fungi and actinomycetes [8, 9]. There are many microbial sources accessible for protease production, only a few are considered as commercial producers [10]. Among them, fungi are well known sources of extracellular enzymes. Protease production by fungi belonging to genera *Aspergillus* [11, 12, 13], *Rhizopus* [14], *Mucor* [13] and *Penicillium* [15, 16, 17] have been studied extensively. Extracellular proteases from fungi can be produced by both submerged fermentation (SmF) & solid state fermentation (SSF).

The growth medium used in SmF is generally optimized using the one-variable-at-a-time method. In this method all variables except one are kept constant, while the optimum level of the testing variable is determined. There is no single defined medium for the optimum protease production from microorganisms as every organism has its own special conditions for maximum enzyme production [18].

To establish a successful fermentation process for protease production, it is essential to optimize the media components such as carbon, nitrogen and inducer source and physical factors such as inoculum size, pH, temperature, aeration and growth time [16].

Microbial alkaline proteases dominate commercial applications with a significant share of about 25% of the total worldwide sales of enzymes for laundry detergent applications. Protease based detergent preparations enables the removal of proteinaceous material from the stains and improves washing effectively with in a shorter duration [19]. The first detergent containing the bacterial enzyme was introduced in 1956 under the trade name BIO-40. Currently, many commercial detergent produces used in the market are serine proteases produced by *Bacillus* strains. However, fungal alkaline proteases are advantageous due to the ease of downstream processing to prepare a microbe-free enzyme [20]. Recently, there has been increasing research about utilization of fungal proteases in detergent industry. However, only a few fungal strains are exploited commercially.

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The present study is aimed to optimize suitable medium and culture conditions for extracellular protease production by *Penicillium* sp. LCJ228 under submerged fermentation and removal of blood stains from the cotton fabric using partially purified protease.

## 2. MATERIALS AND METHODS

### 2.1 Organism and culture maintenance

The mould *Penicillium* sp. LCJ228 was isolated from soil samples using Potato Dextrose Agar (PDA) plates. The culture was screened for protease activity based on the hydrolysis on the skimmed milk agar medium. The culture was maintained on PDA slants and was stored at 4 °C.

### 2.2 Fermentation medium and culture conditions

Production of protease by submerged fermentation was studied in five different basal medium. Medium 1 Sarker *et al.*, 2013) [21] g/L : Glucose, 10; Peptone, 10; K<sub>2</sub>HPO<sub>4</sub>, 1; MgSO<sub>4</sub>, 0.2; Na<sub>2</sub>CO<sub>3</sub>, 5; Medium 2 [19] g/L : Glucose, 2; Yeast extract, 1; K<sub>2</sub>HPO<sub>4</sub>, 0.1; KH<sub>2</sub>PO<sub>4</sub>, 0.1; Casein, 1.5; Medium 3 [22] g/L: Glucose, 10; Gelatin, 10; K<sub>2</sub>HPO<sub>4</sub>, 1; MgSO<sub>4</sub>, 0.5; Medium 4 [23] g/L: Glucose, 10; Casein, 5; Yeast extract, 5; MgSO<sub>4</sub>, 0.2; K<sub>2</sub>HPO<sub>4</sub>, 1; Na<sub>2</sub>CO<sub>3</sub>, 10; Medium 5 [24] g/L: Yeast extract, 5; Peptone, 5; Glucose, 10; Casein, 20. About 100 mL of medium was dispensed into 250 mL conical flask and autoclaved at 121 °C for 15 mins.

A pinch of nalidixic acid was added to avoid bacterial growth. One mycelial disc was inoculated into the conical flask, under sterile condition and incubated on a rotary shaker at 120 rpm. 10 mL of the culture filtrate was taken every 48 hrs and centrifuged at 10,000 rpm for 10 mins. The supernatant was used as a crude enzyme solution for protease activity.

### 2.3 Time course of protease production

The growth curve was obtained by inoculating culture in 250 mL Erlenmeyer flasks containing 100 mL of production medium. The flasks for each sampling were incubated on a rotary shaker at 120 rpm. The experiment was designed for 10 days starting from the lag phase to stationary phase.

### 2.4 Optimization of fermentation conditions

Under submerged fermentation, nutritional factors such as different carbon sources namely glucose, sucrose, fructose, maltose, starch and lactose, different nitrogen sources namely peptone, yeast extract, ammonium chloride, sodium nitrate and different inducer sources including casein, groundnut oilcake, mahua oilcake, black gram husk and red gram husk were studied. The effect of their concentration ranging for 5 to 30 g/L, influencing the production of protease was optimized by incorporating one-factor-at-a-time method. Similarly, the influence of pH (4 to 9), inoculum size (1 to 5 g/L), static and shaking conditions were also studied.

### 2.5 Protease assay

Protease activity was determined spectrophotometrically by using casein as substrate. 500 µL of 0.5% (w/v) of casein (pH 7) in 0.2 M phosphate buffer of 300 µL (pH 7) and 200 µL crude enzyme extract were mixed together and incubated at room temperature for 10 mins. After incubation, the enzyme reaction was terminated by the addition of 1 mL of 5% (w/v) trichloroacetic acid (TCA) [25]. The reaction mixture was then centrifuged to separate the unreacted casein at 10,000 rpm for 15 minutes. To 1 mL of supernatant, 5 mL of 0.4 M Na<sub>2</sub>CO<sub>3</sub> and 1 mL of 3-fold diluted Folin Ciocalteu's reagent were added and mixed well. The resulting solution was incubated in the dark for 30 mins at room temperature and absorbance was measured at 660 nm. The protein content was estimated by following the method described by Lowry *et al.*, [26] using bovine serum albumin as the standard.

### 2.6 Partial purification of protease

The cell-free culture filtrate of *Penicillium* sp. culture was collected by centrifugation after 4 days of growth. The extracellular enzyme was precipitated by the addition of ammonium sulphate (70 %) to saturation. The mixture was incubated overnight at 4 °C and separated by centrifugation at 10,000 rpm for 30 mins.

### 2.7 Enzyme characterization

Molecular weight of the protease was determined by Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS - PAGE). After electrophoresis, the gel was stained with CBB R250 and protein bands were visualized under an illuminator.

For zymography, 0.5% (w/v) gelatin was co-polymerized with the running gel and samples were then loaded onto the gel without heating followed by electrophoresis with 100 V at room temperature. Following electrophoresis, the gel was incubated in 2.5% (v/v) Triton X-100 (renaturing solution) for 30 mins at room temperature with gentle agitation, then was decanted and replaced with developing buffer (50 mM Tris, 0.2 mM NaCl, and 5 mM CaCl<sub>2</sub>, pH 8.0) and incubated at 37 °C for at least 20 hrs. The gel was stained with Coomassie Brilliant Blue G-250 in 0.5% (w/v) for 30 mins and the band was observed under illuminator.

### 2.8 Destaining of blood stains

The efficiency of the protease to remove blood stains was analyzed by the stain removal test. Three pieces of uniform sized cotton fabric were stained with goat blood, dried at room temperature and fixed with 1% (v/v) formaldehyde. The stained fixed fabrics were immersed separately in the crude enzyme, commercial detergent and precipitated protease for different time intervals ranging from 10 to 120 mins at room temperature and examined for stain removal. The same procedure was followed for the control without the enzyme and detergent.

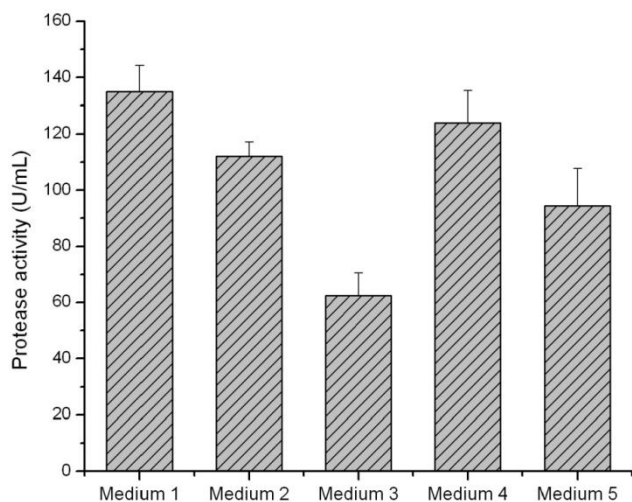
## 2.9 Statistical analysis

The statistical method used in this study was the analysis of variance (ANOVA) using the software SPSS 11.5 and all data are results of duplicate experiments.

## 3. RESULTS AND DISCUSSION

Fungal proteases have several advantages over those produced from other sources and it also find applications in many industries. Proteases production is an inherent capacity of all fungal isolates, however only those species that produce substantial yield of extracellular proteases are considered to be industrial important [27]. Due to its vast applications, promising protease producing fungi are to be screened for the production of protease having desirable properties. Protease producing fungi showed clear zone of casein hydrolysis on skimmed milk agar [28], which was taken as the basis of screening in the present study. The preliminary screening revealed that *Penicillium* sp. LCJ228 was the most potent protease producer as it showed highest radius of hydrolysis zone. Similarly, there are earlier reports on protease production by *Penicillium* sp. [17,29,30].

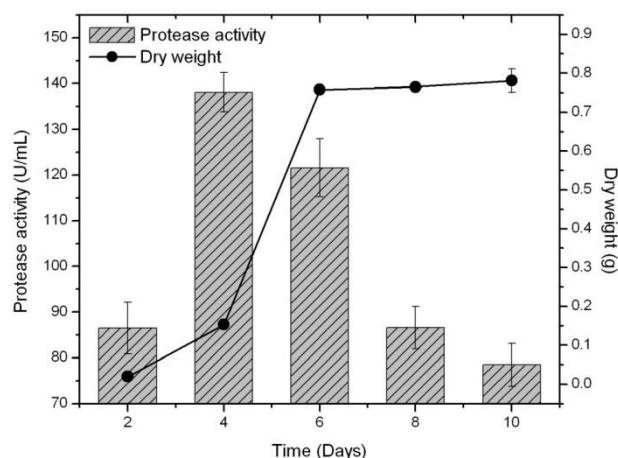
Under submerged fermentation, media components for maximum protease production have been found to be different for each species. Therefore, the required chemical constituents and their concentrations have to be optimized accordingly [31]. In the present study, the production of protease by *Penicillium* sp. LCJ228 was carried out using five different media. Significant variations in biomass and protease activity were observed with different media tested. Initiation of fungal growth was observed after the 2nd day of inoculation. Among the five media tested, Medium 1 favoured maximum protease production with 134.2 U/mL of protease activity on the 4th day (Figure 1).



**Fig. 1:** Effect of different culture media on protease production by *Penicillium* sp. LCJ228 on the 4<sup>th</sup> day.

The growth pattern and protease production by *Penicillium* sp. LCJ228 was studied and results are presented in Figure 2. The protease activity was the maximum on the 4th day

and increase in biomass was observed on the 6th day. After 6th day, a decline in protease activity was observed with increased in time biomass. Earlier it was reported that increased incubation time and declined protease activity may due to the depletion of nutrients in the production medium [32]. This could be due to altered physiology resulting in the inactivation of secretory machinery of the enzymes. In previous studies, maximum protease production by fungal cultures was reported on 4th to 9th day of incubation [12,33].



**Fig. 2:** Growth curve and protease production by *Penicillium* sp. LCJ228 in Medium 1

Several strategies have been adopted for the optimization of both physical factors as well as media components. In the present study one-factor-at-a-time method was employed for the optimization of protease production under submerged fermentation. Extracellular protease production in microorganisms is strongly influenced by medium components, e.g. variation in carbon and nitrogen ration, presence of easily metabolizable sugar, such as glucose [10] and metal ions [34]. It has also been reported that the optimization of medium components is carried out to maintain a balance between the different medium components and thus minimizes the amount of unutilized components at the end of the fermentation process [35].

In the present study, the ability of *Penicillium* sp. LCJ228 grown using various carbon sources indicated their specific utilization by the fungus. Among the mono-, di- and polysaccharides used in this study, glucose was the best carbon source for maximum protease production with 130.8 U/mL of protease activity at 5% level of significance (Table 1). This may be due to the reason that glucose is a monosaccharide and it is readily available for the metabolism of the fungus for enzyme production. Fructose and glucose proved to be the best carbon sources for improving the productivity of protease by *Aspergillus flavus* and *Aspergillus terreus* [36]. Similar results were also observed by Srinubabu *et al.*, [37] and Tremacoldi and Carmona [38]. It has also suggested that different carbon sources have different influences on extracellular enzyme production by different strains [39,40].

**Table 1:** Effect of different carbon and nitrogen sources on the protease production by *Penicillium* sp. LCJ228 on the 4<sup>th</sup> day.

Factors	Protease activity (U/mL)
<b>Carbon source (10 g/L)</b>	
Starch	61.2 ± 1.09
Fructose	120.2 ± 1.75
Lactose	100.0 ± 3.87
Maltose	104.0 ± 4.01
Glucose	130.8 ± 2.47
Sucrose	43.6 ± 1.92
<b>Nitrogen source (10 g/L)</b>	
Yeast extract	250.2 ± 4.94
Peptone	204.8 ± 6.82
NH <sub>4</sub> Cl <sub>2</sub>	34.4 ± 1.76
Na <sub>2</sub> NO <sub>3</sub>	154.6 ± 3.32

The effect of glucose concentration showed that 20 g/L of glucose enhanced maximum protease activity (156 U/mL) at 5% level of significance and declined beyond optimum concentration (Table 2). This decreased protease production at higher concentration of glucose may be due to the repression exerted by excessive amount of metabolizable sugar in enzyme production.

**Table 2:** Effect of concentrations of glucose and concentrations of yeast extract on the protease production by *Penicillium* sp. LCJ228 on the 4<sup>th</sup> day.

Factors	Protease activity (U/mL)
<b>Glucose concentration (g/L)</b>	
5	111.8 ± 5.17
10	139.2 ± 2.23
15	158.8 ± 3.19
20	156.8 ± 9.12
25	146.6 ± 2.98
30	141.2 ± 3.21
<b>Yeast extract concentration (g/L)</b>	
5	209.2 ± 6.09
10	210.2 ± 3.12
15	280.8 ± 1.56
20	238.0 ± 9.12
25	248.6 ± 11.0
30	237.2 ± 5.95

Effect of different nitrogen sources and their concentration on the production of protease by *Penicillium* sp. LCJ228 was studied using different organic (peptone and yeast extract) and inorganic nitrogen sources (ammonium chloride, ammonium nitrate). The results showed that the growth and protease secretion was the maximum with organic nitrogen sources. Addition of yeast extract to the medium significantly ( $p < 0.05$ ) enhanced maximum protease production with 250.2 U/mL of protease activity (Table 1). Similar observation was also made earlier where organic nitrogen sources were found to be effective than inorganic nitrogen sources for protease production [41]. Gnanadoss and Devi [17] suggested that organic nutrients are less expensive and also supply necessary minerals and vitamins required for protease production. It was also proved that yeast extract is a suitable nitrogen source which stimulates protease production in *Aspergillus terreus* [42]. The production of protease possibly was enhanced due to the high protein and amino acid components in the yeast extract.

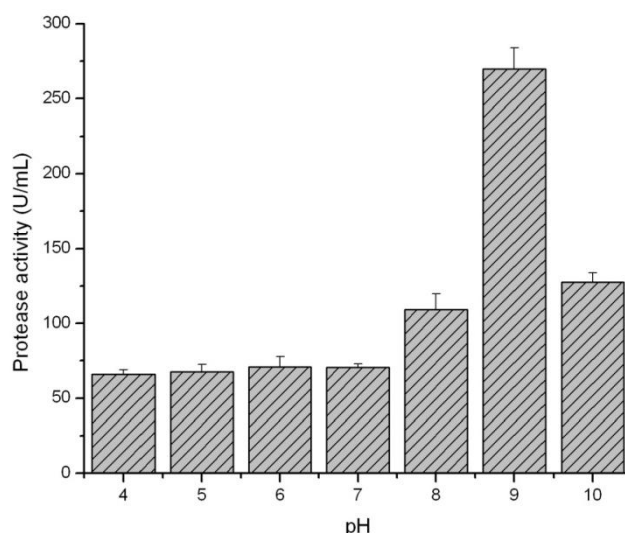
The effect of different concentrations of yeast extract was also studied and results showed that 15 g/L of yeast extract

enhanced maximum protease production (280.8 U/mL) at 5% level of significance (Table 2). The inhibitory effect of yeast extract on the protease production was observed with an increasing concentration and this may be due to nitrogen metabolite repression.

Casein is one of the best chemical inducer for maximize the production of protease. In the present study, the production of protease by *Penicillium* sp. LCJ228 was optimized using both chemical and natural inducer. On the 4th day of incubation, 5 g/L of casein (chemical inducer) showed maximum protease production with 220.1 U/mL. In contrast, addition of powdered black gram husk (natural inducer) to the medium showed maximum (243.4 U/mL) protease activity and this was statistically significant at 5% level (Table 3). The yield of protease with addition of black gram husk powder to the medium was higher than casein amended medium. Higher protein content of the black gram husk could have possibly induced protease production by *Penicillium* sp. LCJ228. Kirankumar *et al.*, [43] also reported that black gram husk significantly induced the production of protease by *Trichoderma viridae*. Similar observation was also reported earlier where agricultural wastes could be used as the natural inducers for protease production which can reduce the cost of the medium [44].

**Table 3:** Effect of inducers on the protease production by *Penicillium* sp. LCJ228 on the 4<sup>th</sup> day.

Inducer	Protease activity (U/mL)
Without inducer	129.8 ± 5.09
Casein	220.1 ± 7.01
Groundnut oilcake	156.8 ± 2.87
Mahua oilcake	172.4 ± 9.13
Red gram husk	177.0 ± 10.0
Black gram husk	243.4 ± 5.28

**Fig. 3:** Effect of different medium pH on the protease production by *Penicillium* sp. LCJ228 on the 4<sup>th</sup> day.

Enzyme production by the fungal culture is strongly dependent on the medium pH. It influences most of the enzymatic processes and the transport process of diverse of components across cell membrane [45, 46]. In the present study, pH 9 (alkaline

pH) favoured maximum protease production by *Penicillium* sp. LCJ228 with 269.8 U/mL of protease activity at 5% level (Figure 3). Similar results were also reported by Palanivel *et al.*, (2013) in *Aspergillus* strain KH17 [47]. It has also been reported that fungal proteases are active at neutral pH and alkaline pH around pH 7 to pH 11 [48].

Inoculum size also plays an important role in protease production. In the present investigation, the effect of inoculum size on the production of protease by *Penicillium* sp. LCJ228 was studied. 2 g/L inoculum size significantly ( $p < 0.05$ ) enhanced maximum protease production with 256.6 U/mL of protease activity (Table 4). It was observed that protease activity decreased with as the inoculum size increased. It was earlier reported that the decline in enzyme production with high inoculum size was related to high biomass which is responsible for the reduction of the enzyme [49]. Similarly, lower protease production at lower inoculum size is probably due to less biomass which is insufficient to utilize the production medium for maximum protease production.

**Table 4:** Effect of inoculum size on the protease production by *Penicillium* sp. LCJ228 on the 4<sup>th</sup> day.

Factors	Protease activity (U/mL)
Inoculum size (g/L)	
1	142.9 $\pm$ 5.09
2	256.0 $\pm$ 3.41
3	211.5 $\pm$ 3.77
4	207.2 $\pm$ 6.12
5	180.0 $\pm$ 4.68

The production of protease in shaking condition (263.3 U/mL) was better when compared to static cultures (110.6 U/mL). This was proved in the present study, shaking condition showed maximum protease activity on the 4th day of incubation. In the fermentation of aerobic culture, the oxygen affects the production of enzymes due to the changes in the metabolic pathway and metabolic fluxes [50]. According to Ducros *et al.*, [51], the respiration rate of the aerobic culture is dependent on the dissolved oxygen. This is due to physiological alteration in cell metabolism [52].

Protease production by *Penicillium* sp. LCJ228 in optimized and original medium was studied. The optimized medium showed a maximum amount of protease production of 261.5 U/mL on the 4th day when compared to the original medium which showed a protease activity of 107.2 U/mL. Nearly, 2.4 fold increases in protease production was observed in optimized conditions.

The steps involved in purification of protease produced by *Penicillium* sp. LCJ228 are summarized in Table 5. After ammonium sulphate precipitation, the enzyme showed 1.3-fold increase in activity with 146 U/mg of specific activity of protein and 69% yield. Muthulakshmi *et al.*, [49] observed 2-fold purification with 66% of recovery by ammonium sulphate precipitation of protease from *Aspergillus flavus*. The molecular weight of the ammonium sulphate protease was determined by SDS-PAGE as ~66 to 70 kDa. The activity of protease was also

observed by Zymography. Prominent clearing zone was observed indicating that the gelatin was hydrolyzed by the protease obtained by ammonium sulphate precipitation. The Zymogram also showed the presence of three isoenzymes.

**Table 5:** Partial purification of protease from *Penicillium* sp. LCJ228.

Purification Steps	Enzyme activity (U/mL)	Total protein	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	251	2.3	109	1.0	100
Ammonium sulphate precipitates (70%)	175	1.2	146	1.3	69

The performance of protease from *Penicillium* sp. LCJ228 in the removal of blood stains from fabric was examined. The precipitated protease from *Penicillium* sp. LCJ228 had the efficiency to remove blood stain completely from the piece of cotton fabric without the aid of any of the detergents (Figure 4). This shows the potential of the enzyme from *Penicillium* sp. LCJ228 in the removal of blood stains. Hence the enzyme can be used as an additive in detergent to enhance its stain removal. This result is in accordance with the earlier reports which also show efficient digestive properties of protease [53]. The alkaline protease from *Penicillium* sp. LCJ228 has a potential application role as an additive in the detergent formulation. The detergent compatibility of the alkaline protease also reported in *Aspergillus* sp. [54]. In the present study, the blood stains were removed in a lesser time when treated with the enzyme.



**Fig. 4:** Removal of blood stains from cotton fabric treated with precipitated protease from *Penicillium* sp. LCJ228 (a control; b commercial detergent wash; c treated with precipitated protease)

#### 4. CONCLUSION

The present study proved that *Penicillium* sp. LCJ228 is an efficient protease producer. Optimization of conditions for protease production under submerged fermentation will help in the large scale production of this promising enzyme. The protease from *Penicillium* sp. LCJ228 also effectively removed blood stains from the cloths than the commercial detergents. Hence the protease from *Penicillium* sp. LCJ228 can be utilized in detergent industries. However, future studies on molecular identification of *Penicillium* sp. LCJ228, and purification and characterization of protease from *Penicillium* sp. LCJ228 would help to extent its applications in various biotechnological and environmental aspects.

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