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PRODUCTION OF ALKALINE PROTEASE FROM *ASPERGILLUS ORYZAE* VIA STATIC LIQUID SURFACE CULTURE TECHNIQUE AND ITS POTENTIAL APPLICATION AS A DETERGENT ADDITIVE

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ABSTRACT

The objective of this study was to investigate the production, purification, and characterization of alkaline protease obtained from *Aspergillus oryzae* ML-1 via static liquid surface culture fermentation. The proteolytic activity of different types of fungi was evaluated by measuring the clear zone on a skim milk agar plate. Based on the results, *Aspergillus oryzae* ML-1 was selected for further study. During optimization of process parameters, the highest protease yield (1091 U/ml) was obtained after 216 h of incubation at 30°C; with initial pH 6.0 and inoculum level, 4%. The enzyme was purified in a procedure involving ammonium sulphate precipitation and Sephadex G-100 size exclusion chromatography. The enzyme was found to have a molecular weight of 33 kDa by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)/zymography and was purified by around 2.03 times with a yield of 63.10%. The K_m and V_{max} values of purified protease were 0.66 mg/ml and 15.60 µM/ml/min, respectively. The results of inhibitors study showed that the enzyme was inhibited 100 % with PMSF indicating its serine nature. While among metal ions, Ca^{+2} and Mg^{+2} ions accelerated the relative enzyme activity up to 136% and 124%, respectively. In addition, the purified enzymes exhibited maximum stability over a broad range of pH 7-11 and temperature 30- 45°C indicating its potential application in detergent formulation for laundry purpose.

Keywords: Process optimization, Purification, Characterization, Protease, *Aspergillus*

INTRODUCTION

Proteases constitute one of the most important groups of industrial enzymes that have extensive applications in pharmaceutical, food, leather, and detergent industries [1,2]. Among the various types of proteases, alkaline proteases are known to account for 60%–65% of the global industrial market [3]. Plants, animals, and microbial sources are employed for protease production. Microbes serve as the preferred source of proteases because of their rapid growth, the limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties [4]. Currently, a large proportion of commercially available enzymes are derived from *Bacillus* species [5, 6]. Filamentous fungi are also used in many industrial processes for the production of enzymes, considering that the produced enzymes are normally extracellular and easy to separate from fermented broth [7] Moreover, enzyme production from fungi is safer than that from bacteria because such enzymes are generally recognized as safe (GRAS) [8, 9]. *Aspergillus* species are known to produce large quantities of enzymes in their growth environment [10]. The cost and properties of an enzyme are the major considerations in its commercial exploitation at industrial level. Recently, solid-state fermentation (SSF) has attracted considerable attention in the production of specific enzymes because it reduces costs by utilizing unprocessed or moderately processed raw materials [11, 12]. Despites its advantages, SSF is often problematic in terms of monitoring process parameters such as pH, temperature, oxygen, and biomass concentrations because of the solid nature of the fermented material. These parameters play a vital role in making fermentation cost-effective at the commercial scale, and any change in these parameters can have a drastic effect on enzyme yield. Thus, the limitations of SSF provide researchers with an impetus to investigate alternative fermentation processes for enzyme production. Static liquid surface culture (SLSC) fermentation is a process whereby a culture is grown on the surface of the growth medium in stagnant conditions (without vigorous shaking or physical agitation). This type of fermentation can easily be exploited for the production of commercially significant enzymes, such as proteases, from various fungus cultures. Recently, SLSC fermentation has been used for enzyme production from *Aspergillus niger* [13].

In the present study, alkaline protease produced from *Aspergillus oryzae* ML-1via (SLCS) fermentation is purified and characterized in order to explore its appropriate use in detergent formulation. In addition, various process parameters are optimized to improve its yield.

Materials and Methods

Proteolytic fungal strain

A fungal strain of *Aspergillus oryzae* ML-1 was obtained from the Microbiology Lab, PCSIR Laboratories Complex, Lahore. The strain was screened on skim milk agar medium to visualize its proteolytic efficiency. The skim milk agar medium was composed of 1.0% skim milk, 0.1% peptone, 0.5% sodium chloride, and 1.5% agar. The pH of the medium was adjusted at 8.0 with 1N HCl/NaOH before sterilization at 121°C for 15 min. Finally, the proteolytic fungal strain was grown on potato dextrose agar (Oxoid) slants at 35°C for 5 days, after which it was maintained at 4°C for subsequent study.

Fermentation process

Next, 50 ml of the growth medium, composed of glucose (10 g/l), yeast extract (5 g/l), meat extract (1 g/l), KH_2PO_4 (2 g/l), NaCl (0.5 g/l), and $CaCl_2$ (0.5 g/l) was inoculated with 2% (v/v) spore suspension ($10^{6–7}$ spores/ml) in a 250-ml Erlenmeyer flask and incubated at 35°C for 10 days in static condition. Thereafter, the sample was filtered using filter paper (Whatman No. 1) and the protease activity was determined in cell-free filtrate. The initial pH of the growth medium was adjusted at 8.0 with 1N HCl/NaOH before sterilization at 121°C for 15 min. The glucose was sterilized separately and mixed into other medium constituents at the time of inoculation under aseptic conditions.

Optimization of process parameters

Various process parameters, such as fermentation time (72–240 h), initial pH (4–9), incubation temperature (20–45°C), and inoculum level (2%–10%, v/v), were investigated to determine the most suitable culture conditions for the maximum yield of protease from *Aspergillus oryzae* ML-1. All these parameters were evaluated individually while keeping the other parameters constant, and each parameter was incorporated at the optimum level in the experiment before optimizing the next parameter. The optimization experiments were conducted in triplicate and the mean values were reported.

Determination of biomass

A known volume of inoculated broth was filtered through pre-weighed filter paper (Whatman No. 1). The cell biomass on the filter paper was washed with distilled water

twice and then dried at 80°C in an oven until constant weight was attained. Then, the filtrate was maintained at 4°C for subsequent study.

Determination of total protein content

The total protein content in the cell-free sample was estimated by the method of Lowry [14] using bovine serum albumin (BSA) as the reference standard.

Determination of protease activity

Protease activity was determined according to the method described by Yang and Huang [15] after slight modification. The reaction mixture, containing 2 ml of 1% casein solution in 0.05M Tris-HCL buffer (pH 8.0) and 1 ml of enzyme solution, was incubated at 40°C for 20 min, and the reaction was then stopped with the addition of 3 ml of 10% trichloroacetic acid. After 10 min, the entire mixture was centrifuged at 10000 rpm for 10 min at 4°C and the absorbance of the liberated tyrosine was measured with respect to the blank sample at 280 nm. One enzyme unit (U) was defined as the amount of enzyme that releases 1 µg of tyrosine per minute under assay conditions.

Purification of protease enzyme

Salt precipitation

The crude enzyme was fractionated via salting out with ammonium sulphate at different concentrations (40%–80%) and then centrifuged at 10000 rpm for 20 min at 4°C in order to obtain the precipitate in pellet form. The protein pellets were then dissolved in minimal volume of 0.1M Tris-HCl buffer (pH 8.0), after which they were dialyzed against the same buffer to remove the salt concentrations.

Gel filtration chromatography

The desalted fraction of protease at 60% saturation with ammonium sulphate was purified via Sephadex G-100 (Pharmacia) column chromatography (1×30 cm) using an FPLC system (Biologic LP, Bio-Rad, USA). The column was equilibrated with 0.05M Tris-HCl buffer (pH 8) and then eluted with the same buffer at a flow rate of 30 ml/h. The fractions showing absorbance at 280 nm were pooled, analysed for protease activity, and lyophilized using a Freeze dryer (Eyela, Japan). The lyophilized preparation was stored at -20°C for further study.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE (12.5%) was performed according to the method described by Laemmli [16] using mini slab gel apparatus (8×8 cm glass plate). The gels were stained with Coomassie Brilliant Blue R-250. Further, medium-range molecular weight proteins (Fermentas) were used as the standard to determine the molecular mass of pure protein.

Characterization of enzyme

Effect of pH on protease activity and stability

The effect of pH on protease activity was determined by incubating the reaction mixture at pH values ranging from 4–12 using the following buffers (0.05M): citrate phosphate (pH 4.0–5.0), phosphate (pH 6.0–7.0), Tris-HCl (pH 8.0–9.0), and Glycine-NaOH (pH 10–12). Then, pH stability was studied by pre-incubating the enzyme at 30°C in buffers of various pH values (4–12) for 30 min. After incubation, the residual activity (%) was measured as per the described procedure.

Effect of temperature on protease activity and stability

The effect of temperature on enzyme activity was determined by incubating the reaction mixture at different temperatures (30–60°C). The thermostability of the enzyme was investigated by pre-incubating it in the same buffer at various temperatures (40–80°C) for 30 min, and the residual activity was measured according to the standard assay procedure.

Effect of inhibitors and metal ions on protease activity

To investigate the effect of inhibitors and metal ions (each at 5 mM), the purified protease was pre-incubated with various inhibitors, such as phenyl methyl sulphonyl fluoride (PMSF), di-isopropyl fluorophosphate (DFP), TPCK, TLCK, *p*-chloromercuric benzoate (*p*CMB), and ethylene diamine tetra acetic acid (EDTA), and metal ions, such as Ca^{+2}, Mg^{+2}, Na^{+2}, Hg^{+2}, Cu^{+2}, Al^{+3}, Ni^{+2}, Ba^{+2}, and Co^{+2}, for 30 min at 30°C. Then, the remaining activity was measured routinely while taking 100% activity in the absence of inhibitors and metal ions.

Enzyme kinetics

The K_m and V_{max} values for alkaline protease were calculated from the Lineweaver-Burk plot (double reciprocal plot) constructed by plotting reciprocals of various concentrations of the substrate on the x-axis and reciprocals of the enzyme velocity on the y-axis.

Statistical Analysis

The data represented herein are expressed as mean ± SD. Significance is presented as the results of Duncan's multiple range test in the form of probability ($P \leq 0.05$) values, which were obtained using CoStat software.

RESULTS AND DISCUSSION

Initial screening study

A clear zone on a skim milk agar plate is a basic tool for visualizing the proteolytic efficiency of any strain prior to its use in the fermentation process for protease production. Therefore, a fungal strain, *Aspergillus oryzae*

ML-1 was grown on skim milk agar (pH 10), and complete hydrolysis of skim milk was observed after incubation at 35°C for 96 h, as shown in Figure 1.

Optimum process parameters studies

3.2.1 Effect of incubation period

The results of the present study showed that protease production and cell biomass increased with the incubation period. Maximum production of protease (623.3 U/ml) and cell biomass (1.58 g/l) was observed at 216 h of incubation (Figure 2). Thereafter, enzyme yield and cell biomass were found to gradually decrease with further increase in the incubation period, clearly suggesting that the enzyme produced by *Aspergillus oryzae* ML-1 was growth-associated and secreted into the growth medium as a primary metabolite. These results are in accordance with the observation made by Paranthaman *et al*. [17]. However, different incubation periods for the optimum yield of protease have been reported previously [18,19]. Such types of dissimilarities in incubation time could possibly be due to the different microorganisms and fermentation processes employed for protease production.

Effect of initial pH

Extracellular pH strongly influences many enzymatic processes as well as the transport of various components across the cell membrane, which in turn supports growth and product development [20]. Therefore, the effects of the initial pH (4–9) on the growth of *Aspergillus oryzae* and alkaline protease production were investigated. The results showed that the highest alkaline protease yield (861 U/ml) and growth (2.05 g/l) were recorded at an initial pH of 6 (Figure 3). However, a further increase in the initial pH decreased protease yield and growth. Maximum protease production has been reported in different pH ranges (5–10) from various *Aspergillus* species in previous studies [21,22]. All these findings indicate that the pH requirements for proper growth and product formation vary from species to species and even in different strains of the same species isolated from different habitats.

Effect of incubation temperature

Temperature is considered as one of the key parameters to be controlled in any bioprocess [23]. In the present study, the effects of the incubation temperature indicate that the organism grew well over a wide range of temperatures, whereas maximum protease yield (1090 IU/ml) and growth (2.9 g/l) were noted at 30°C (Figure 4). The variation in temperature above or below the optimum value (30°C) led to a decline in enzyme production and growth. Similar observations

have been reported by some other researchers, who observed maximum protease yield and growth at 30°C from various *Aspergillus* species [22,24]. All these findings clearly indicate that most of the protease-producing *Aspergillu*s species are mesophilic in nature.

Effect of inoculum level

A lower inoculum level may not be sufficient for the proper growth of microbes, whereas a higher level may cause competitive inhibition [25]. Therefore, optimization of inoculum level is crucial for boosting enzyme yield in fermentation processes. In the present study, maximum protease production (1091U/ml) and growth (3.67 g/l) were obtained with an inoculum level of 4%, as shown in Figure 5. However, a further increase in inoculum level decreased the enzyme yield. This can be attributed to the rapid depletion of nutrients in the growth medium, resulting in decreased enzyme yield as well as metabolic activity. A wide range of inoculum levels have been optimized for the maximum yield of proteases in previous studies [18, 19].

Purification of alkaline protease

The purification of crude alkaline protease produced from *Aspergillus oryzae* ML-1 is summarized in Table 1. Initially, crude enzyme solution was precipitated with ammonium sulphate, and 60% saturation level increased the protease activity by around 1.41 times with 80.36% recovery. The enzyme suspension at 60% saturation level was then subjected to gel filtration chromatography on a Sephadex G-100 column for further purification. The elution profile yielded a well-resolved single peak, indicating enzyme purification (Figure 6). The active fractions of this peak were pooled. The protease activity increased by around 2.04 times, with a recovery of 63.10% and specific activity of 449.01 U/mg of protein. The purity of the enzymes was further confirmed by SDS-PAGE. A unique single protein was observed in the gel, indicating homogeneity of the purified protease (Figure 7). The molecular weight of the purified *Aspergillus oryzae* protease was estimated to be 33 kDa using molecular weight markers. In general, a wide range (25–50 kDa) of molecular masses produced by various *Aspergillus* species has been reported in other studies [26–28].

Characterization of purified protease

Effect of pH on enzyme activity and stability

The present enzyme was found to be active over a broad range of pH (7–11) with 100% activity at pH 11, indicating the alkaline nature of the enzyme (Figure 8). However, with further increase in pH (pH 12), the

activity reduced to 78%. An investigation of the pH stability showed that the enzyme was variably stable over a wide range of pH. However, maximum stability (>91%) was exhibited in the pH range of 8–11. At pH 7 and 12, the enzyme showed >78% stability. The remarkable stability over a wide range of pH and alkaline nature of this enzyme makes it a promising candidate for detergent application. Several studies have reported alkaline proteases with broad pH activities and stabilities, obtained from bacterial sources; however, alkaline protease production from fungus culture has not been investigated extensively thus far. An alkaline protease produced from *Aspergillus oryzae* AWT20 was found to be active over a pH range of 7–9, with maximum activity at pH 9 [26]. In another investigation, a purified alkaline protease produced from *Aspergillus niger* showed maximum activity at pH 10 [29].

Effect of temperature on enzyme activity and stability

The activity of any enzyme over a broad range of temperatures reflects its desired characteristics for commercial application. In the present study, the alkaline protease produced from *Aspergillus oryzae* ML-1 has been found to be active and stable over a wide range of temperatures. However, maximum protease activity and stability were observed at 40°C when tested at pH 11 using casein as a substrate (Figure 9). A similar observation has been reported previously for alkaline protease produced from *Aspergillus niger* [29]. As far as thermostability concerned, the enzyme retained 100% stability up to 45°C, and above this temperature, its stability decreased gradually. Minimum stability (35%) was observed at 60°C, and below this temperature, the enzyme retained >50% stability, indicating its novelty as an appropriate candidate for detergent formulation. Sharma *et al.* [26] reported 10% stability of *Aspergillus oryzae* alkaline protease at 55°C.

3.4.3 Effect of inhibitors and metal ions on protease activity

The effect of various inhibitors and metals ions on alkaline protease produced from *Aspergillus oryzae* is summarized in table 2. The results show that the enzyme activity was inhibited by 100% with the serine protease inhibitor phenylmethyl sulphonyl fluoride, indicating the serine nature of the protease. Similar findings on inhibition by PMSF have been observed for alkaline protease produced from *Aspergillus oryzae* [26]. Another serine inhibitor, di-isopropyl fluorophosphate (DFP), reduced the protease activity by up to 97%, while metalloprotease

inhibitors, such as EDTA and 1,10-phenanthroline, did not show any significant effect on protease activity. Yin *et al*. [30] reported a protease of cysteine nature produced from *Aspergillus oryzae*. All these findings indicate a diverse nature of proteases produced from various species or even from the same species isolated from different habitats.

In general, the presence of suitable metal ions plays an important role in maintaining active conformation of an enzyme against thermal denaturation [31]. Therefore, the recognition of suitable metal ions has a significant impact on enzyme applications. In the present study, it was found that Ca^{+2} and Mg^{+2} ions accelerated the relative enzyme activity by up to 136% and 124% respectively, while other metal ions slightly reduced the enzyme activity. Nehra *et al*. [32] found Mg^{2+} to be an activator of the alkaline protease enzyme produced from *Aspergillus* species. Similarly, the presence of Ca^{2+} is also known to activate protease activity by increasing thermostability [33,34].

Kinetic studies

The kinetic parameters K_m and V_{max} of alkaline protease produced from *Aspergillus oryzae* ML-1 were determined using the Lineweaver-Burk plot under different concentrations (5–25 mg/ml) of casein as a substrate (Figure 10). The results show that the K_m and V_{max} values of the alkaline protease were 0.66 mg/ml and 15.60 µM/ml/min, respectively. Siala *et al*. [35] estimated K_m of 1.02 mM and V_{max} of 2.2 µmol/min for the caseinolytic activity of protease produced from *Aspergillus niger* I1. Muthulakshmi *et al*. [36] 2011 reported K_m and V_{max} of 0.6 mg/ml and 60 U/mg, respectively, for alkaline protease produced from *A. flavus*. Yin *et al*. [30] estimated K_m and V_{max} of value 0.12 mM and 14.29 µmol/min, respectively, for acidic protease produced from *Aspergillus oryzae* BCRC 30118 using haemoglobin as a substrate. Low values of K_m indicate that the enzyme has high affinity to the substrate and dissociates rarely before the substrate is converted into the product. Consequently, the K_m value of the alkaline protease produced from *Aspergillus oryzae* ML-1 further proves that the enzyme may preferably be used in industrial applications, such as industrial protein hydrolysis, owing to its catalytic efficiency.

Figure 1: Visualization of proteolytic efficiency of *Aspergillus oryzae* ML-1 grown on skim milk agar plate after incubation at 35°C for 96 h

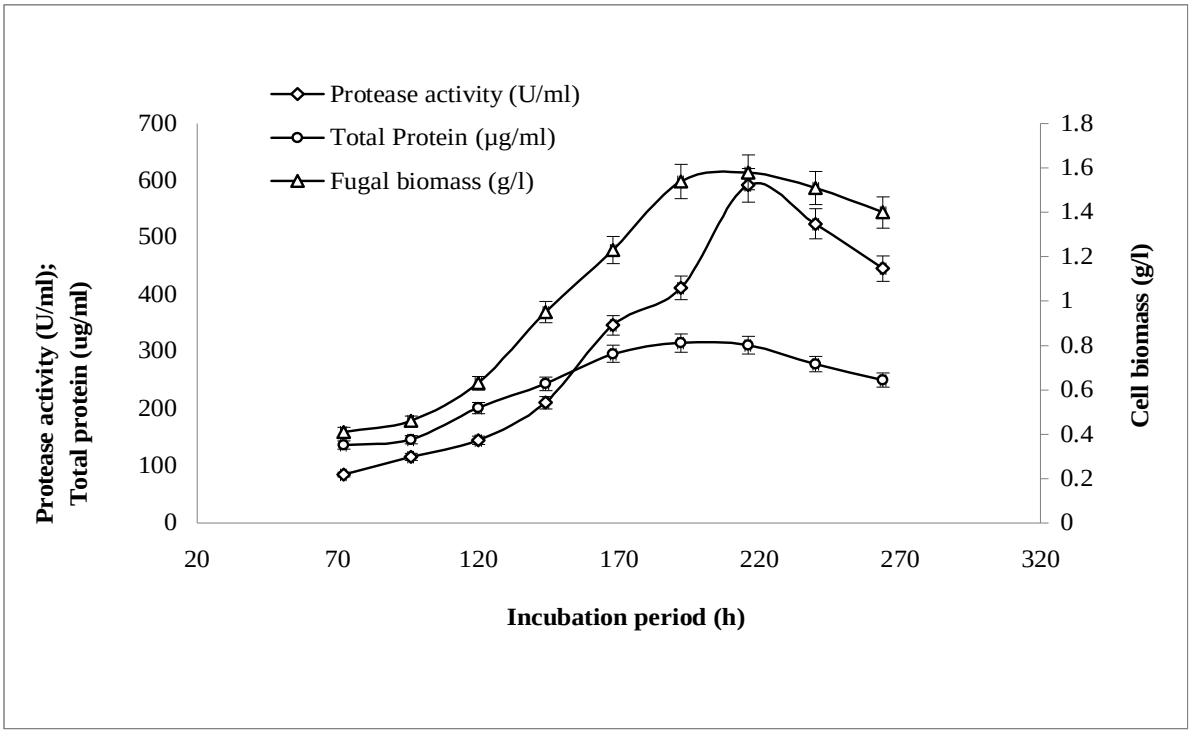

Figure 2: Effect of incubation period on alkaline protease production from *Aspergillus oryzae* ML-1 via static liquid surface culture fermentation (initial pH, 8.0; temperature, 35°C).

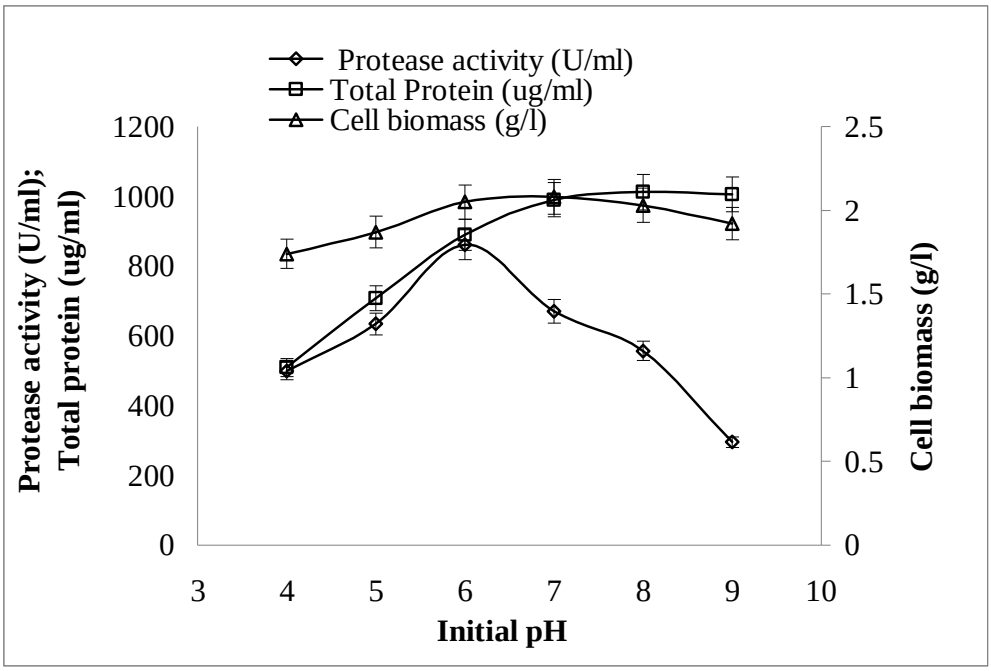

Figure 3: Effect of pH on alkaline protease production from *Aspergillus oryzae* ML-1 via static liquid surface culture fermentation (temperature, 35°C, Incubation period, 216 h)

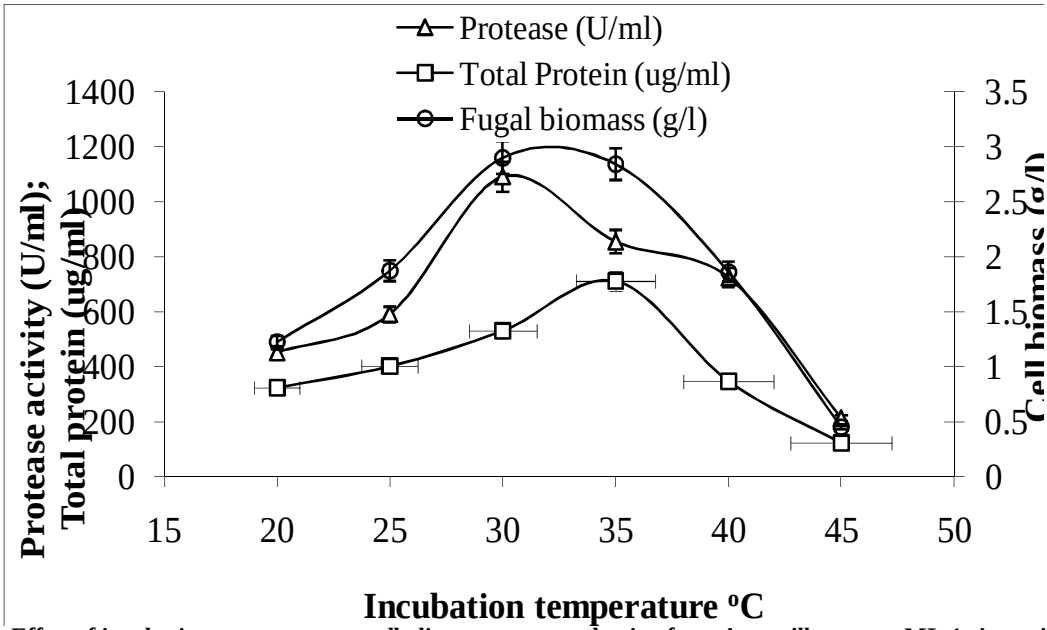

Figure 4: Effect of incubation temperature on alkaline protease production from *Aspergillus oryzae* ML-1 via static liquid surface culture fermentation (initial pH, 6.0; Incubation period, 216 h)

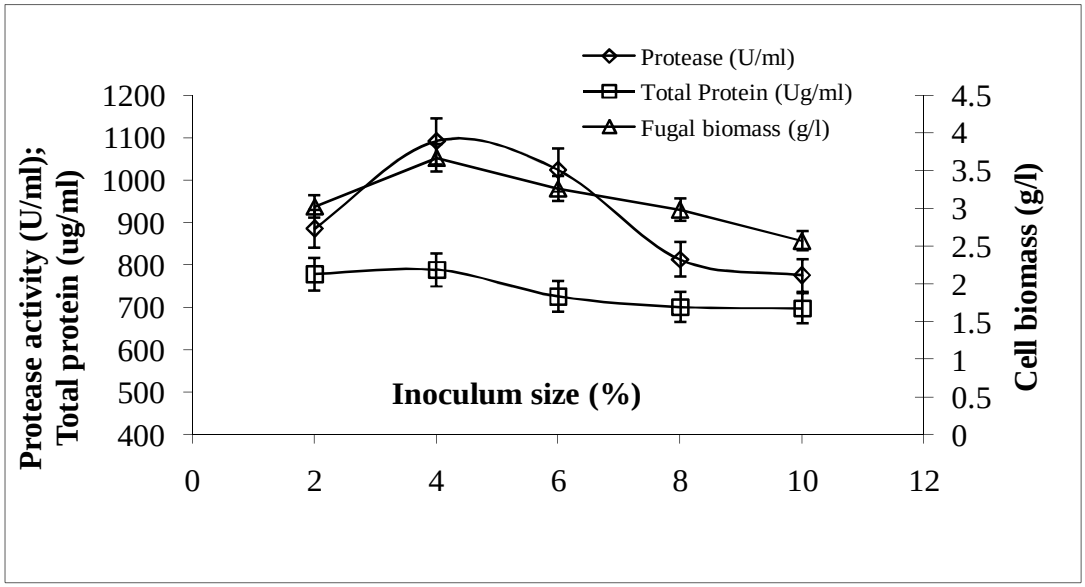

Figure 5: Effect of inoculum level on alkaline protease production from *Aspergillus oryzae* ML-1 via static liquid surface culture fermentation (initial pH, 6.0; incubation period, 216 h)

Table 1: Summary of purification process of alkaline protease

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification Fold	Yield (%)
Crude enzyme	353600	1606.6	220.12	1	100
Ammonium sulphate precipitation (60%)	16500	53.10	310.73	1.41	80.36
Sephdex-G-100	7004.50	15.60	449.01	2.04	63.10

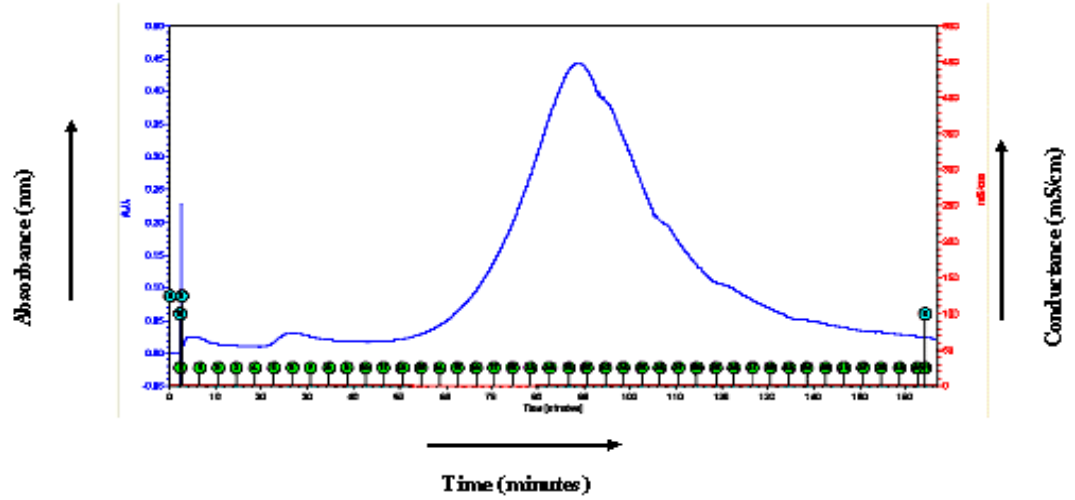

Figure 6: Elution profile of alkaline protease from Sephadex G-100 column (1×31 cm) by FPLC.

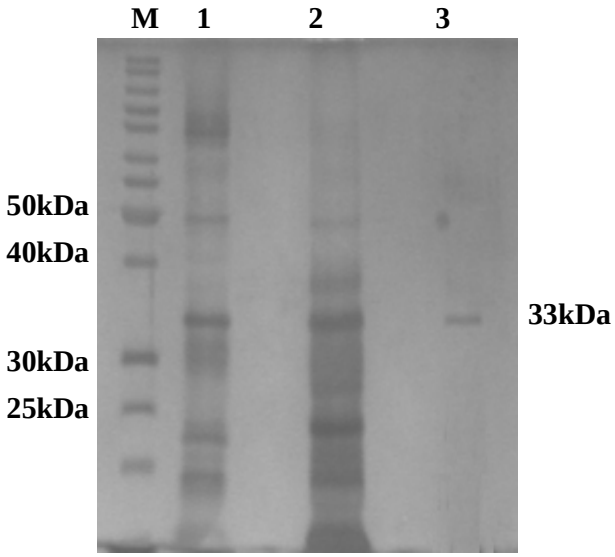

Figure 7: SDS-PAGE pattern of purified proteases after (A) staining with Coomassie R-250 and (B) silver staining. Electrophoresis was performed using 15% SDS-PAGE. Lane M: molecular mass standards; Lane A, crude enzyme; Lane B, dialyzed sample; Lane C, gel filtration chromatography fraction. A 33-kDa band of protease was observed in all the three samples

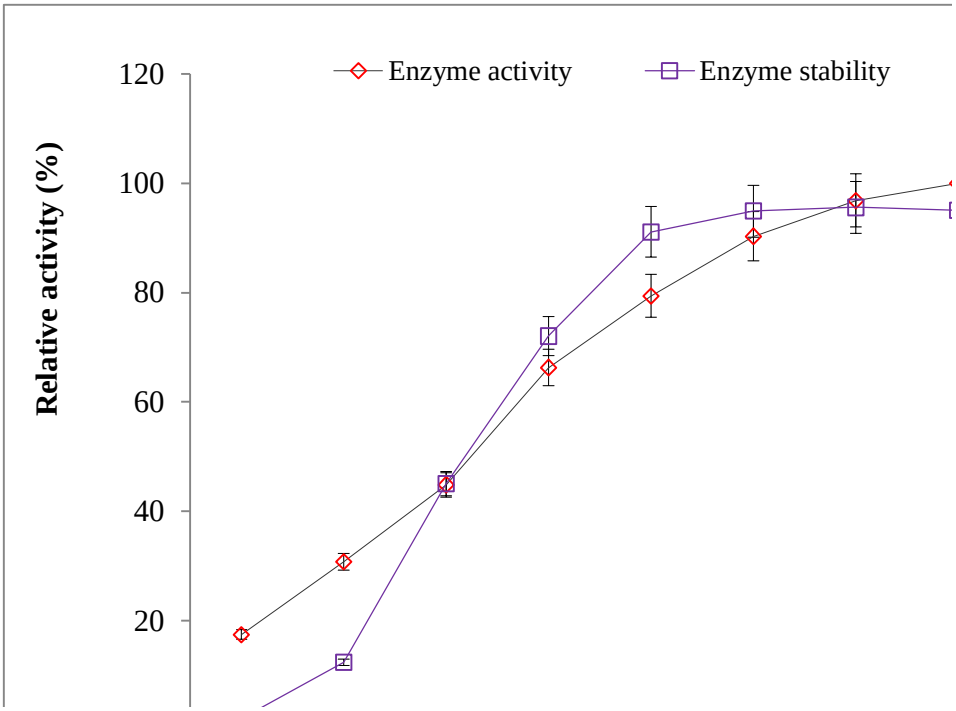

Figure 8: Effect of different pH levels on activity and stability of protease produced from *Aspergillus oryzae* ML-1. The bars represent ± SD.

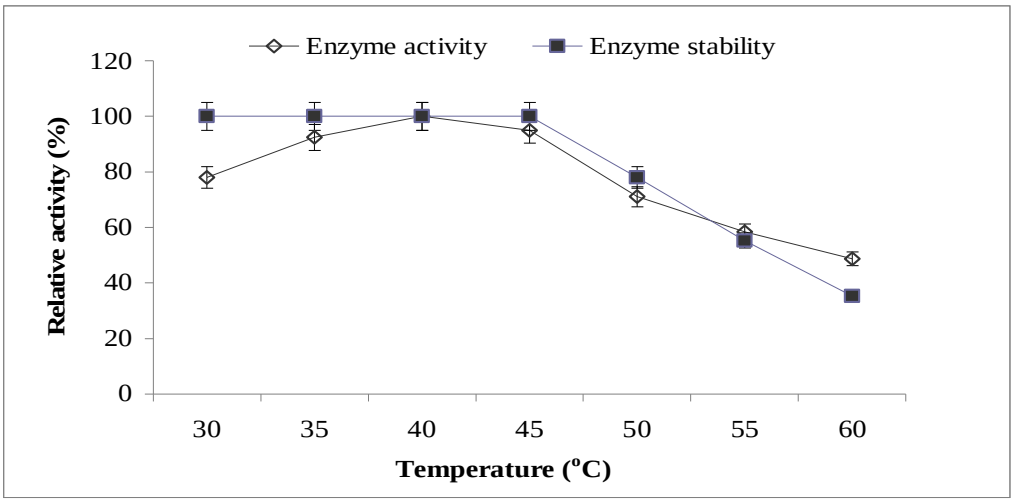

Figure 9: Effect of temperature on activity and stability of protease produced from *Aspergillus oryzae* ML-1. The bars represent ± SD

Table 2: Effect of inhibitors and activators on the relative activity of alkaline protease produced from *Aspergillus oryzae* ML-1

Inhibitor/activator	Relative activity (%)
Control	100
PMSF	0
DFP	11
I, 10 phenanthroline	94
ρCMB	96
EDTA	91
Ca^{2+} ($CaCl_2$)	136
Mg^{+2} ($MgCl_2$)	124
Na^{2+} ($NaCl_2$)	94
Hg^{2+} ($HgCl_2$)	42
Cu^{2+} ($CuCl_2$)	97
Al^{3+} ($AlCl_3$)	95
Ba^{2+} ($NiCl_2$)	89
Co^{2+} ($CoCl_2$)	92

PMSF= Phenyl methyl sulphonyl fluoride; DFP= di-isopropyl fluorophosphate; I, 10 phenanthroline; ρCMB= para chloromercuric benzoate; EDTA= Ethylene diamine tetra acetic acid. The concentration of all the inhibitors and metal ions was adjusted at 5 mM.

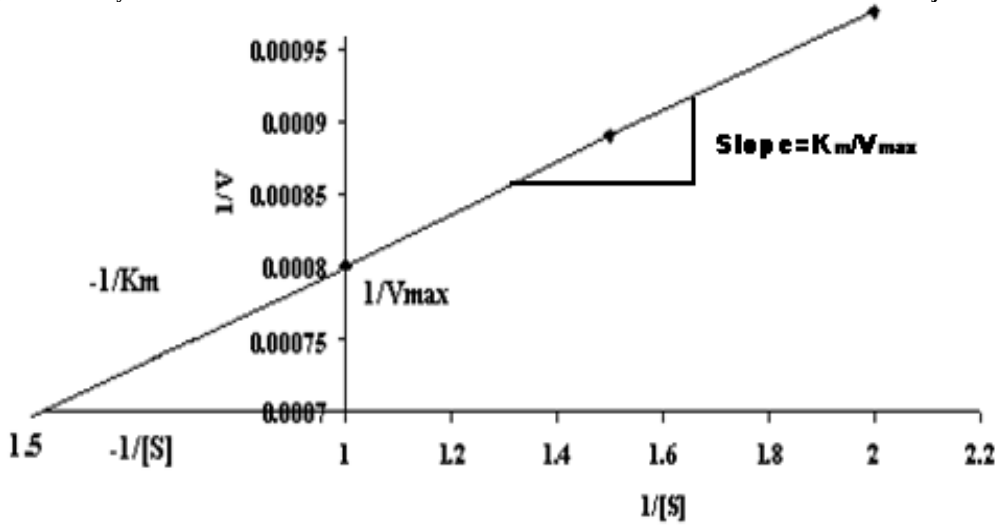

Figure 10: Lineweaver-Burk plot showing K_m and V_{max} values for alkaline protease under varying substrate (casein) concentrations (5–25 mg/ml).

CONCLUSION

From the results, it has been concluded that a suitable fermentation process for enzyme production and characterization of enzyme in purified form played a pivotal role in exploring its commercial significances. In present study, Maximum protease production (1091U/ml) was obtained by employing *Aspergillus oryzae* ML-1 in surface culture fermentation after process parameters optimization. Protease enzyme produced by *A. oryzae* was purified up to homogeneity level with ammonium sulphate precipitation (60 %) and gel filtration through Sephadex G-100 chromatography. The enzyme was purified up to 2.04 fold with a specific activity of 449.01U/mg and 63.10 % recovery after complete purification. The molecular weight of the enzyme was estimated to be 33 kDa by SDS-PAGE. The inhibition study showed that PMSF completely inhibited the enzyme activity, suggesting its metallic nature. The purified enzyme showed desirable properties such as high activity and stability at broad ranges of pH (7-11) and temperature 40^{o}C. All these properties indicate the potential of commercial exploitation of the *Aspergillus oryzae* ML-1 alkaline protease in detergent formulations.

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