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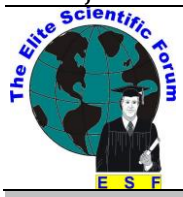

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RESEARCH ARTICLE

Production of a Serine Alkaline Proteinase from *Bacillus Subtilis* by Using Low-Cost Substrate and Its Purification

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ABSTRACT

Proteases from *Bacillus subtilis* were produced by using low-cost substrate and purified for their eco-friendly applications. The enzyme was purified using four-step purification: Ammonium sulphate precipitation, dialysis, ion-exchange chromatography and gel filtration chromatography. The molecular weight of the purified enzyme was estimated to be 65 kDa on SDS-PAGE. It exhibited optimum activity at broader temperature range and maximum at 60 ˚C under alkaline pH environment and fermentation time 48 hours of continuous shaking at 220 revolution per minute. Specific activity of the purified protease was increased to 3 folds. These characteristics renders potential use of the proteases in biotechnological and industrial sectors.

INTRODUCTION

Proteases (EC 3:4, 11-19, 20-24) are the enzymes that cause lysis of other proteins or alike themselves in catalytic pattern, dream up the biggest industrial tribe of enzymes consisting almost 2% of the human genome (Butt et al., 2018). They widely exist in the digestive portion of living species, imparting a very decisive role in protein consumption and digestion. The production of proteases by microbes has industrial implications. *Bacillus* (*B.*) *subtilis* produces both neutral and alkaline proteases (Dhandapani and Vijayaragavan, 1994). Proteases produced by *B. subtilis* have wider specificity than those from animal origin (Shaheen et al., 2008). The *B. subtilis* is obtained from soil and known as hay bacillus/ grass bacillus. The organism exhibit rod-like shape and survive under extraordinary difficult environmental conditions. *Bacillus* are facultative anaerobes (Pant et al., 2015). Very encouraging use of proteases are revealed in therapeutics of different disorders like cancer, cardiac diseases and inflammations of wounds (Raza et al., 2015; Abdel-Naby et al., 2020).

After the selection of hyper producing strain, a low-cost substrate, skimmed milk, was chosen for the study. Selection of the substrate is very important in the fermentation not only to obtain the optimal yield but also at a lower cost. Optimization of substrate is primarily required in fermentation because different organisms react differently for every substrate. Skimmed milk is cheaper as compared to many other sources. The purification of enzymes was carried out by ammonium sulfate precipitation after dialysis followed by ion-exchange chromatography and/- gel filtration. This study aimed to produce serine proteases from *B. subtilis* on pilot scale followed by purification and characterization and thus leading to possible applications in the industry.

MATERIALS AND METHODS

Microorganism

The *B. subtilis* was procured from Molecular Biology laboratory (MBL), Department of Biochemistry, University of Agriculture, Faisalabad-Pakistan. The organism was sub-cultured on Luria Broth agar slants at 37°C to produce proteases by following the methodology described previously (Butt et al., 2018).

Production and activity assay

Proteases were produced using low-cost skimmed milk as a substrate under fermentation condition/ parameter were already optimized including pH 6.5 to 9.5; temperature 30-50 ˚C; incubation period (24-72 hours); substrate level (0.5-2%); yeast sludge (100-400 µL); ammonium sulphate (0.2-0.5%); urea (0.1-0.25%) and cane molasses (0.3-0.10%) along with continuous shaking condition at 220 rpm. Protease activity was determined spectrophotometrically at 275 nm (Kumar and Vats, 2010).

Purification of protease enzyme

The purification of crude enzyme was done by precipitation by the addition of 80% ammonium sulphate. The pellet after precipitation was dialyzed and all portions of the desalted samples and sediments were assayed for proteases. Purification followed by dialysis of the proteases was carried out by ion-exchange chromatography (IEC) as described by Borah et al. (2012). The prepared column was washed with NaOH/ water and then HCl/ water to achieve neutral pH. The column was equilibrated with buffers of various concentrations until the eluted solution attained a pH of 7.3. One mL of desalted sample was injected on top of the column with the help of a micropipette. The sample was eluted with 50 mM 0.2 M phosphate buffer (pH 7.3) maintaining with constant drop rate of the elute. One hundred fractions with 2 mL each were collected for further experiments of eluted from 50 mM, 100 mM, 125 mM and 150 mM buffers (pH 7.3). IEC was followed by size exclusion chromatography**.** Sephadex G-75 (Pharmacia) was prepared as described by Zia et al. (2007) and Iqbal et al. (2018) and equilibrated with phosphate buffer (pH 7.3). The buffer retained with the column, was removed through outlet, leaving a thin layer of it at the top of column. The 10 mL of sample (pooled fractions) having maximum specific activity was introduced into the column and eluted with phosphate buffer 0.2 M (pH 7.3) at fixed drop rate. Fifty fractions of 2 mL each were collected, and absorbance was noted at 275 nm. A chromatogram was prepared, and the peaks were subjected to proteases assay.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The samples from different purification steps were subjected to SDS-PAGE (Laemmli, 1970) on 10% polyacrylamide slab gels with 0.1% sodium dodecyl sulphate. The protein bands were visualized by staining with Coomassie brilliant blue. The gels were documented on Gel Documentation and Analysis system after destaining (Syngene, UK). The gels were preserved in 7.5% glacial acetic acid solution (ice cold) and washed with ddH_2O. Finally, the gels were preserved in cellophane sheets (Bio-Rad, USA).

Protease activity

Proteases activity was determined by using skimmed milk as substrate by adopting the method of Butt et al., (2018).

Protein estimation

The protein isolated by above-described methods was quantified by Bradford assay using bovine serum albumin (BSA) as standard (Bradford, 1976).

RESULTS AND DISCUSSION

Production of proteases from *B. subtilis*

Proteases were produced form *B. subtilis* using submerged fermentation method and various conditions were optimized in continuation to our previous studies (Butt et al., 2018, 2019).

Kinetic investigations of produced proteases showed lower value of K_M = (0.2) demonstrated good association between substrate and the enzyme. The nature of the reaction mechanism was found to be the first order because [S] was much less than K_M. PMSF inhibition proved that the proteases produced were serine proteases Butt et al., (2019).

Purification of proteases

A mixture of crude proteases obtained from the production was purified by chromatographic procedures after ammonium sulphate precipitation and dialysis. The enzyme activity was determined after each step (Table 1).

Subsequently, further purification of the enzyme was performed by DEAE- cellulose ion-exchange chromatography. Through the column, 100 fractions were collected and assayed for the proteases (Fig. 1, 2). Pooled samples with highest activity were run on Sephadex G-75 for gel filtration chromatography. After gel filtration chromatography the samples were run on SDS-PAGE.

For procuring alkaline/microbial protease obtained from the cell-free supernatant, ammonium sulphate precipitation (80%) and dialysis were done that resulted in 1.1fold purification with ~21% recovery. The specific activity of the dialysate was 11.33 U/mg of protein that is comparable to some previous reports. Ozcelik et al., (2014) recorded purification steps of proteases from *B. pumilus* D3 with 1.61 folds along with yield of 21%. .

The purification of crude enzyme via DEAE cellulose column chromatography provided 2.3 folds rise in purity with 16% recovery of proteases. Asker et al., (2013) produced 5.42 folds increase in purity with 18% recovery of proteases. Ozcelik et al. (2014) also reported 18% yield of proteases with 5.05 folds increase in proteases from *B. pumilus* D3.

The gel filtration chromatography yielded 3.0 folds rise in activity of proteases from *B. subtilis* with 3.18% recovery. Current results are in accordance with those reported by Ahmed et al. (2011) who obtained 1.49 folds rise in proteases with 3.11% recovery in yield. Muthulakshmi et al. (2011) demonstrated 5.8 folds increase in proteases with the recovery of 3.2%.

Table 1: Summary of the purification of proteases from *B. subtilis*

Steps	Protease production (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	502	50	10.24	1	100
After $(NH_4)_2SO_4$	102	9	11.33	1.1	20.3
Ion exchange Chromatography	80	3	26.6	2.3	15.9
Gel filtration	16	0.2	80	3.0	3.18

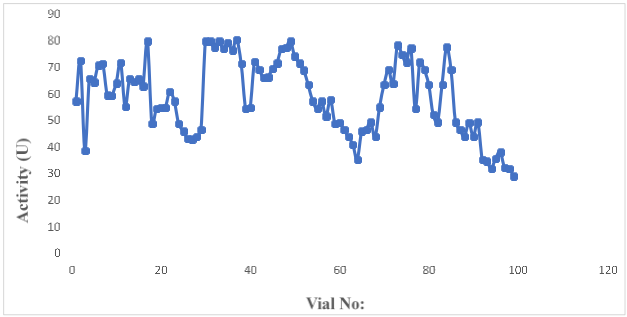

Fig. 1: Chromatogram of ion-exchange chromatography for proteases from *B. subtilis* produced on skimmed milk as a substrate.

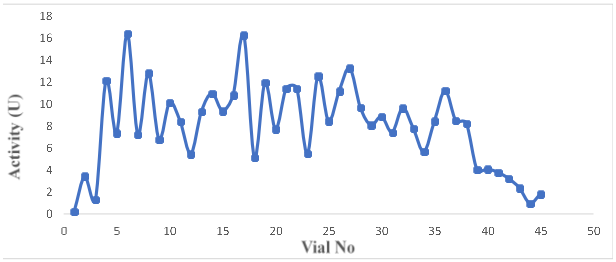

Fig. 2: Chromatogram of gel filtration chromatography of proteases from *B. subtilis* produced on skimmed milk as a substrate.

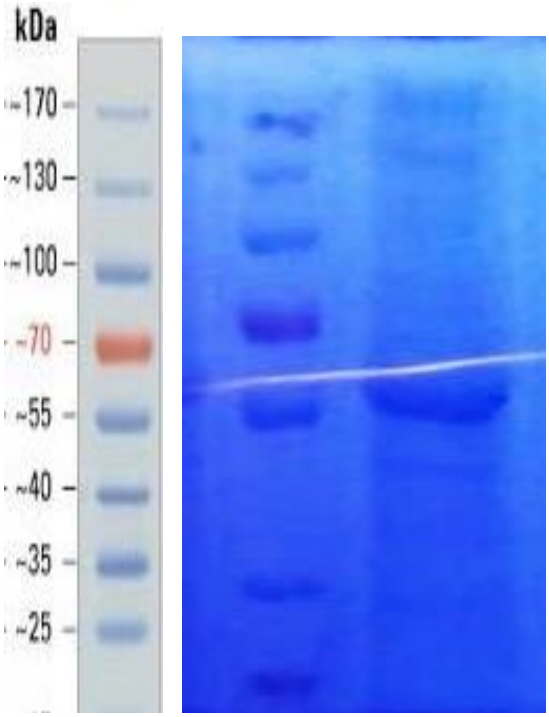

Fig. 3. SDS- PAGE for the crude and purified proteases from *B. subtilis* produced on skimmed milk as a substrate. Lane 1 (Crude proteases, lane-2. (Purified Proteases)

Sookkheo et al. (2000) obtained 2.6 folds rise in the yield of proteases with 7% yield of proteases from *B. stearothermophilus* TLS33. Therefore, our results demonstrate that the proteases were purified to comparable levels and could be utilized further.

Molecular weight determination

The purified proteases were resolved on SDS-PAGE and showed a single band of molecular weight of 65 kDa relative to the standard molecular weight markers (Fig. 3). Our results are supported by Sookkheo et al. (2000), Sharma and De (2011) and Mothe and Sultanpuram (2016) who found the molecular weights of proteases to be 66 kDa, 63 kDa and 71 kDa, respectively. Bajaj et al. (2014) also confirmed by SDS-PAGE analysis the size of a proteases to be 60 kDa. Proteases showed highest activity at 50 °C and pH 8. Previous investigation also showed the molecular weight of proteases to be 61.4 kDa and having optimal temperature 40 °C and pH 5 (Liu et al., 2013). Similar findings were presented by Thuy and Bose (2011) and Pillai et al. (2011) after SDS-PAGE of proteases showed the molecular weight of 67 kDa and 66 kDa, respectively. Molecular weights of purified proteases in the range 44 - 50 kDa was reported by Prasanth et al. (2016).

Conversely, our findings differ from Shinde et al. (2012), Lagzian and Asoodeh (2012) and Asker et al. (2013) who reported the molecular weight of purified protease in the range of 20- 32 kDa.

It was concluded that *B. subtilis* has profound capacity for the production of proteases. In conclusion, *B. subtilis* holds virtuous activity of proteases. The investigation has also systematized the production parameters of bacilli for maximal production of the proteases. The optimal pH and temperature of the proteases were found at 7.5 and 60 °C, respectively. Purified serine protease has a strong potential to be applied in cuisine industry, detergent industry, and pharmaceutical/leather industry because of its stoutness at elevated optimal temperature/pH as well as high Vmax and substrate specificity.

Authors' Contribution

KYB performed the research and data analysis. SK conducted the part of research. MIG planned the research. MAK performed the data Analysis. AYB performed the purification of proteases. Final draft of the manuscript was approved by all authors.

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