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Research Article

KERATINOLYTIC PROTEASE PRODUCTION BY *BACILLUS CEREUS* STRAIN PS03 UNDER SUBMERGED FERMENTATION: OPTIMIZATION AND CHARACTERIZATION

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Abstract

In the present study, chicken feather powder was screened for its application as the substrate for the production of keratinolytic protease by *Bacillus subtilis* strain PS03. *Bacillus subtilis* produced a high level of keratinolytic protease using chicken feather powder as substrate. With feather powder as substrate, physical factors such as incubation time, pH and temperature were optimized for increased keratinolytic protease production by *Bacillus subtilis*. The enzyme production was enhanced when using maltose as carbon source and yeast extract as nitrogen sources. SDS-PAGE analysis indicated the molecular weight of 46 kDa of the partially purified keratinolytic protease. The keratinolytic protease enzyme was stable over a pH range of 6 – 9 and temperature range of 35 - 50°C with maximum activity at pH 9 and 40°C. Based on the results, the use of feather powder as substrate for keratinolytic protease production is cost effective and is easy to scale up. Considering the availability and cost, chicken feather powder is considered as an ideal substrate for keratinolytic protease production in an industrial point of view.

Keywords: Feather powder; *Bacillus subilis* PS03; keratinolytic protease

Introduction

Microorganisms serve as the production warehouse for many industrially important enzymes (Mitidieri *et al*., 2006). Especially, proteolytic enzymes find their application in a wide range of industries such as pharmaceutical, food, animal feed, bioremediation, leather, detergent, paper, textiles, etc. The selection of microorganisms having potential enzyme activity will have substantial impacts at present and in future (Vermelho *et al*., 2013). Keratin, the structural protein component of hair and feathers is an insoluble protein resistant to microbial degradation due to the tight packing of the protein chain (Mazotto *et al*., 2011). Keratin can be degraded by keratinolytic enzymes produced by certain species of the genus *Bacillus* (Cedrola *et al*., 2012) and fungi (Gradisar *et al*., 2005).

The keratinolytic proteases play an important role in the enzymatic production of feather meal and feed additives for cattle (Odetallah *et al*., 2005), amino acid production, leather and detergent industries, etc. (Mazotto *et al*., 2011; Cedrola *et al*., 2012). After enzyme treatment the insoluble feather can be transformed into either fertilizers or feeds and also used in the amino acid production. *Bacillus subtilis* is one of the most important industrial bacterial species that produces a variety of enzymes such as amylases, cellulase and proteases (Morya *et al*., 2012). Enzymes from *Bacillus* sp. have been used in food industries for several decades. Even though their proteases have been studied, reports on keratinolytic protease production by *Bacillus subtilis* are not in-depth (Lopes *et al*., 2011). With these backdrops, the present study aimed to evaluate the keratinolytic enzyme production by *Bacillus subtilis* PS03 in submerged fermentation, its optimization and characterization.

Methodology

Bacterial Strain

The proteolytic bacteria, *Bacillus subtilis* strain PS03 isolated from tannery effluent contaminated soil was used for the present investigation.

Screening for Keratinolytic Enzyme

The proteolytic bacteria were streaked on to keratin-agar plates consisting (g/l): keratin, 10; peptone, 5; yeast extract, 1; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; Na_2CO_3, 10; agar-agar, 15 (Han et al., 2012). After 48 h of incubation,

keratinolytic protease production was evidenced by the appearance of clear zone around the colony.

Keratinolytic Enzyme Production

About a loopful culture of *Bacillus subtilis* PS03 was inoculated on to inoculation medium which consists (g/l): peptone, 5; beef extract, 1.5; yeast extract, 1.5; sodium chloride, 5. In order to examine the keratinolytic enzyme production, 1 ml of the above inoculum was added to 100 ml of culture medium containing (g/l): feather powder, 30; peptone, 5; $MgSO_4$, 0.1; yeast extract, 5; K_2HPO_4, 1 (Han et al., 2012). For analyzing feather degradation, 0.1 % of grounded feather powder was added to the basal medium. The medium was incubated at 37°C for 48 h. The culture medium was then centrifuged and the cell free supernatant was used for assay procedures.

Assay for Keratinolytic Enzyme

The keratinolytic activity was assayed by adding 100 µl of cell free culture filtrate to 400 µl of azokeratin (10 mg/ml) dissolved in 0.1 M phosphate (pH 8.0) and incubated for 15 min at 50ºC. The reaction was then stopped by adding 2 ml of trichloroacetic acid and the mixture was centrifuged at 10000 rpm for 5 mins. The absorbance of the supernatant was measured at 440 nm. One enzyme unit can be defined as the amount of enzyme required to increase the absorbance of 0.01 (Aruna Devi and Lakshmi, 2014). Protein concentration was determined by Bradford method (Bradford, 1976) using bovine serum albumin as standard.

Optimization of Process Parameters

The process parameters involved in the production of keratinolytic enzymes were optimized further. The factors considered were fermentation period (12 - 120 h), pH (6 - 11), temperature (30 - 60°C), carbon sources (1%, w/w) (glucose, sucrose, maltose, lactose, xylose, and starch), and nitrogen sources (1%, w/w) (peptone, yeast extract, ammonium nitrate, urea, and casein).

Partial Purification of Keratinolytic Enzyme

The keratinolytic protease enzyme produced under optimized conditions was partially purified using ammonium sulphate precipitation and dialysis. The cell free culture filtrate was subjected to 60% saturation of ammonium sulfate to obtain partially purified keratinolytic protease. The crude enzyme was obtained by centrifuging the precipitate at 10000 rpm for 20 min. The precipitate obtained was dissolved in 10 mM sodium phosphate buffer, and dialyzed overnight using the same buffer. The partially purified keratinolytic protease enzyme was collected, concentrated and stored at -20°C.

SDS-PAGE Analysis

The protein purity and molecular weight of the partially purified keratinolytic protease was determined using SDS-PAGE electrophoresis (Laemmli, 1970).

Characterization of Keratinolytic Enzyme

The effect of pH on the activity and stability of the keratinolytic enzyme was assayed by determining the enzyme activity after incubation of the enzymes at different pH (6-11) for 1 h. Similarly, the effect of temperature on the keratinolytic activity and stability was determined by determining the enzyme activity after incubation of the enzymes at 30–90°C for 1 h in a constant-temperature water bath. The residual keratinolytic activity and stability was estimated quantitatively using spectrophotometer.

Results and Discussion

The *Bacillus subtilis* strain PS03 isolated from tannery effluent contaminated soil was used in the present investigation. The bacteria were identified using 16S rRNA sequence analysis and the nucleotide sequence were submitted to the Genbank under the accession number KT160016. This study was aimed to screen the bacteria for producing keratinolytic enzyme using keratin-agar plates. The bacterial strain was shown to produce appreciable level of keratinolytic protease enzyme. The ability of *Bacillus subtilis* PS03 to produce enhanced level of keratinolytic protease could offer tremendous potential for the development of biotechnological methods for the hydrolysis of feather waste (Moreira-Gasparin *et al.*, 2009).

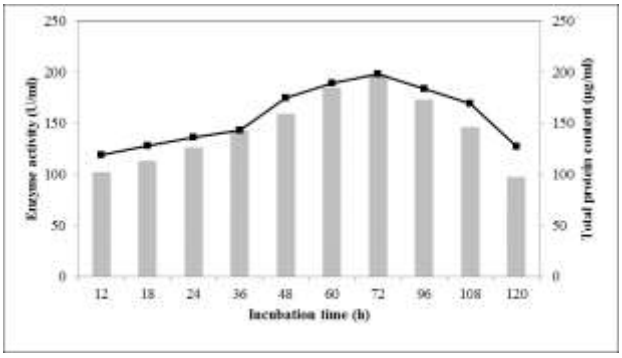

Fig. 1: Time course study on keratinolytic protease production by *B. subtilis* PS03

The effect of incubation time on keratinolytic protease production was studied for a period of 120 h at 37°C and reached maximum enzyme activity during 72 h of incubation (Fig. 1). The results have also shown that beyond 72 h, the enzyme activity decreased considerably. These results obtained are in accordance with the observations made by George-Okafor and Mike-Anosike (2012) with *Bacillus* sp. Since pH influences the metabolic activity of bacterial cell, it was optimized. The keratinolytic protease production was observed between the pH range of 6 – 11 and the enzyme production was maximum at pH 9 and beyond which, substantially decreased (Fig. 2). Based on the observations, the pH of the production medium was kept at 9 in subsequent experiments. Prakasham *et al*. (2006) also found pH 9 as optimum pH for keratinolytic protease production by *Bacillus* sp. Next to pH, temperature plays an important role in the enzyme production. Among the tested

temperatures, 40°C favored the keratinolytic protease production (Fig. 3). Temperature is one of the important factors responsible for the enzyme production as most of the substrates are heat sensitive. Similar trend of results were observed by Sivakumar *et al*. (2012).

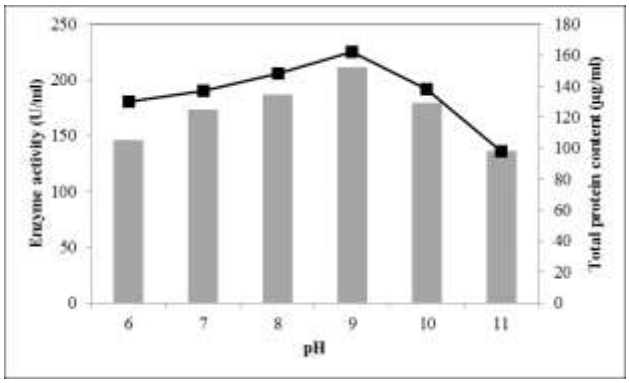

Fig. 2: Effect of pH on keratinolytic protease production by *B. subtilis* PS03

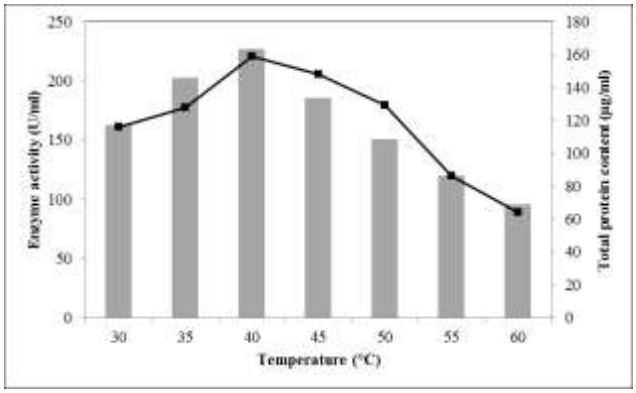

Fig. 3: Effect of temperature on keratinolytic protease production by *B. subtilis* PS03

The results revealed that the carbon and nitrogen sources have a major influence on the yield of keratinolytic protease. Among the carbon sources, maltose favored the maximum keratinolytic protease production followed by lactose, glucose, sucrose, xylose and starch, respectively (Fig. 4). Since, maltose is the simple sugar it can be easily utilized by the bacteria for their metabolic activities and enzyme production. The results obtained were in accordance with reports of Ellaiah *et al*. (2002) and Vijayaraghavan *et al*. (2012). Similarly, among the nitrogen sources tested, yeast extract favored maximum keratinolytic protease production followed by peptone, ammonium nitrate, casein and urea (Fig. 5). Results of similar trends were observed by Pandey *et al*. (2000) and Prakasham *et al*. (2006) who have observed the production of keratinolytic protease using different bacteria.

The keratinolytic protease present in the cell free culture filtrate was precipitated with 70% saturation of ammonium sulfate and dissolved in Tris–HCl buffer. The crude keratinolytic proteases were dialyzed and concentrated. The molecular weight of the partially purified keratinolytic protease enzyme was determined using SDS–PAGE analysis. The partially purified enzyme migrated as a major band with an apparent molecular weight of 46 kDa along with few minor bands. Keratinolytic enzymes with similar molecular weights were isolated and characterized by several researchers. The molecular weight of keratinolytic enzyme was less than 50 kDa for *Bacillus cereus* (Sousa *et al*., 2007) and was 39.5 kDa for *B. circulans* (Rao *et al*., 2009).

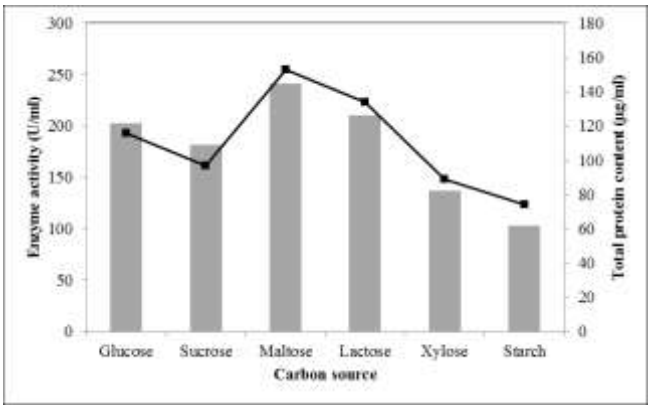

Fig. 4: Effect of carbon sources on keratinolytic protease production by *B. subtilis* PS03

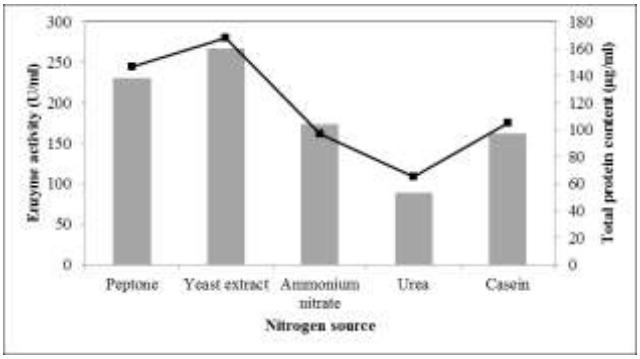

Fig. 5: Effect of nitrogen sources on keratinolytic protease production by *B. subtilis* PS03

After molecular weight determination, the effect of pH on the activity and stability of keratinolytic protease enzyme was studied. The keratinolytic protease was found to be active between the pH range of 6 to 9 and the maximum activity was recorded at pH 9. The relative enzyme activity was recorded a maximum of 100% at pH 9. Beyond which the enzyme activity drops to 39.4 at pH 10 (Fig. 6). The enzyme was found to be stable upto pH 9 for 1 h. Results of similar trend were observed by other researchers when characterizing *Bacillus* sp. producing proteolytic and keratinolytic enzymes (Rajkumar *et al*., 2011; El-Hadj-Ali *et al*., 2007; Haile and Gessesse, 2012). Rao *et al*. (1998) have reported that the commercial bacterial keratinolytic enzymes have pH optima in the range from 8 to 12 and the results of the present study also found in line with his findings.

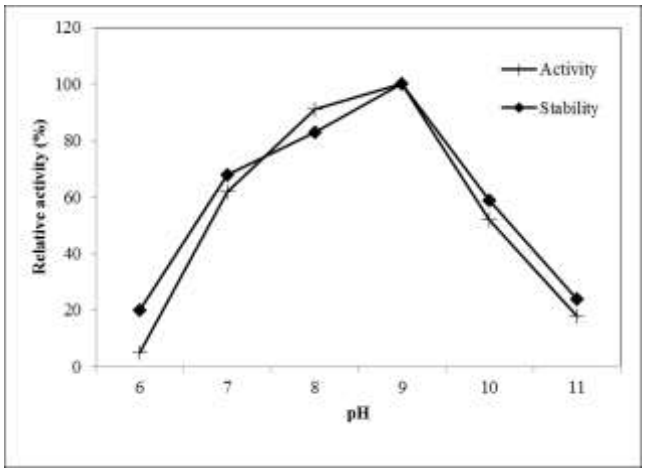

Fig. 6: Effect of pH on the activity and stability of keratinolytic protease

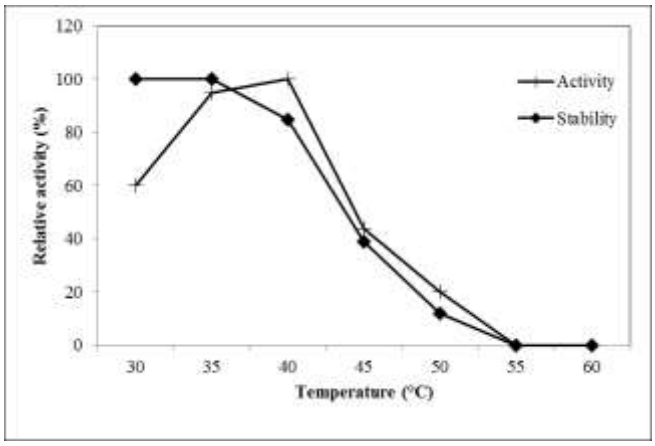

Fig. 7: Effect of temperature on the activity and stability of keratinolytic protease

After pH, the temperature was considered as the factor to characterize the keratinolytic protease enzyme. The maximum keratinolytic protease activity recorded was between 35 and 40°C and the activity decreased rapidly beyond 45°C. In case of stability, the keratinolytic protease was found to be stable up to 40°C for 1 h incubation and lost its activity at 60°C (Fig. 7). Shinde *et al*. (2012) and Haile and Gessesse (2012) also observed similar result while characterizing keratinolytic enzyme using *Bacillus* spp.

Conclusion

In the present study, a keratinolytic enzyme was produced using the feather powder substrate in submerged fermentation. Chicken feather powder is an ideal substrate for keratinolytic protease production in an industrial point of view, considering its cheap cost and availability. The keratinolytic protease from *Bacillus subtilis* PS03 was active in a wide range of pH and temperature. These properties of the keratinolytic protease suggest its suitable application as an additive in the leather industry and in the management of feather waste.

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