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Alkaline Protease Production from Industrial Waste by *Bacillus subtilis* ML-4

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Abstract. The influence of various culture conditions on protease production by *Bacillus subtilis* ML-4 was studied in the presence of growth medium containing poultry feed waste (5%), K_2HPO_4 (0.3%), $CaCl_2$ (0.03%) and $MgSO_4$ (0.015%). Maximum protease production (264.25 ± 1.86 U/ml) was observed at initial pH 9 with 3% (v/v) of inoculum size after 48 h of incubation at 37 °C. The alkaline protease was stable over a broad range of temperature (30 to 60 °C) and pH (8 to 11). However, maximum activity (155.45 U/ml) was observed at temperature 50 °C and pH 10.

Keywords: protease production, *B. subtilis*, poultry feed, detergent formulation

Introduction

Proteases are produced wide spread in nature such as by plants, animals and microorganisms (Rao *et al*., 1998). The microbial sources have advantages over the plant and animals sources due to the ease of growth as well as production. Microbial proteases can be produced by bacteria, fungi and yeast through submerged and solid-state fermentation (Haki and Rakshit, 2003; Anwar and Saleemuddin, 2000; Kumar and Takagiss, 1999). Among various proteases, bacterial alkaline protease is the most important for industrial applications. Alkaline proteases have wide use in industrial processes such as in foods, leather, pharmaceutical and detergent formulations and for cleaning of membranes used in protein ultra filtration (Dayanandan *et al*., 2003; Kumar and Takagi, 1999). Protease is one of the most important industrial enzymes occupying nearly. 60% of the enzyme sales (Adinarayan and Ellaiah, 2003; Beg *et al*., 2003). It is produced mainly by many members belonging to genus *Bacillus* especially, *B. licheniformis*; *B. horikoshii* and *B. sphaericus* (Mehrotra *et al*., 1999). Nowadays, detergent industries are mainly focused on alkaline protease for its use in all types of laundry detergents and in automatic dish-washing detergents for removal of proteinaceous stains (Maurer, 2004).

Cost of the enzyme is the major issue in enzyme production for use in various industrial processes. Therefore, utilization of cheaper industrial waste has significant impact on enzyme utilization. The present study was conducted on enzyme production using the poultry waste as a substrate. Various concentrations of substrate and other process parameters such as pH, temperature, size of inoculum were also studied for the enhancement of enzyme yield so as to make the process cost affective.

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Materials and Methods

Microorganism. *Bacillus subtilis* ML-4 was procured from Microbiology Lab of Food and Biotechnology Research Centre, PCSIR Laboratories Complex, Lahore. *Bacillus subtilis* ML-4 was grown on nutrient agar slant (Oxoid) for 24 h at 37 °C. The culture was then preserved at 4 °C and transferred to new slants after 30 days in order to keep them viable. pH of the nutrient agar was adjusted at 10 with 1N HCl / NaOH before sterilization at 121 °C for 15 min.

Preparation of inoculum. Inoculum was prepared by transferring a loopful of 24 h old *Bacillus subtilis* ML-4 culture to 50 mL of sterile inoculum broth in 250 mL Ehrlenmeyer flask. pH of the medium was adjusted at 10 as mentioned above. The inoculated broth was incubated in a water bath shaker (Eyela, Japan) at 120 rpm and 30 °C for 24 h to prepare the inoculum containing the bacterial load up to $10^{8\text{-}10}$ cfu/mL.

Fermentation of growth medium. The medium used for the production of protease was composed of poultry feed (5%), K_2HPO_4 (0.3%), $CaCl_2$ (0.03%) and $MgSO_4$ (0.015%). pH of the medium was adjusted at 10 as mentioned above. Six percent (v/v) of 24 h old inoculum was transferred to 50 mL of growth medium in 250 mL Erlenmeyer flask under aseptic conditions. The inoculated fermentation medium was incubated in water bath shaker (Eyela, Japan) at 120 rpm at 37 °C for 48 h. Afterwards, the fermentated broth was centrifuged at 4 °C for 10 min at 10,000 rpm to get clear solution.

Effect of substrate concentrations. Eight concentrations of poultry waste 1-8% were optimized for the maximum yield of the enzyme.

Study of process parameters. Various process parameters were studied to optimize the level of each parameter for maximum production of alkaline protease by *Bacillus subtilis* ML-4, which included inoculum sizes of 1-6%, incubation temperatures of 25, 27, 29, 31, 33, 35, 37, 39, 41 and 43 °C and initial pH of 6-12.

Bacterial viable counting. Total viable count was determined according to the method described by Eaten *et al*. (2005). The sample was diluted serially by transferring 1 mL of sample to 9 mL sterile Butterfield's phosphate buffer (pH 7.2). One mL of each dilution was poured into sterilized petri plates, mixed properly with plate count agar (Oxoid) and allowed to settle down. After incubation of plates at 35 °C for 48 h, the bacterial colonies were counted.

Protein determination. Total protein content was estimated according to Lowry *et al*. (1951) using bovine serum albumin as standard.

Determination of protease activity. Protease activity was determined by the method of Yang and Huang (1994). The reaction mixture containing 2 mL of 1% casein solution in 0.05 M glycine-NaOH buffer (pH11) and 1 mL of enzyme solution were incubated at 60 °C for 15 min and then, the reaction was stopped with addition of 3 mL of 10% trichloro-acetic acid. After 10 min the entire mixture was centrifuged at 10000 rpm for 10 min at 4 °C and absorbance of the liberated tyrosine was measured with respect to the blank at 280 nm. One proteolytic unit (U) was defined as the amount of the enzyme that releases 1 µg of tyrosine per min under assay conditions.

Effect of metal ions on thermo stability and pH stability of enzyme. The thermostability of alkaline protease was studied by incubating the enzyme in water bath (Eyla, Japan) at different temperatures from 30 to 70 °C for 1 h in the absence or presence of Ca^{2+}, Cu^{2+} and Mg^{2+} ions at concentration of 5 mM. After the treatment, the enzyme activity was measured according to the standard assay.

pH stability of enzyme was observed at pH 7-14 for 8 h at 40 °C in the presence or absence of Ca^{2+},Cu^{2+} and Mg^{2+} at concentration of 5 mM. Various pH values were adjusted with sodium phosphate buffer (pH 6-7), Tris-HCl buffer (pH 8-9) and glycine NaOH buffer (pH 10-12). After the treatment the enzyme activity was measured according to the standard assay.

Results and Discussion

Effect of substrate concentration. The poultry feed, used as a source of carbon and nitrogen for the production of protease, was studied by cultivating *Bacillus subtilis* ML-4 at different process conditions. The maximum yield (287.41±1.5 U/mL) of protease was found at 5% substrate concentration (Fig. 1). Balassa *et al.* (1978) and Massucco *et al.* (1978) used different concentrations of acid cheese whey as substrate for protease production. Soybean meal was also used as medium for the production of protease (EI-Enshasy *et al*., 2008).

Effect of inoculum size. It is generally necessary to optimize age and size of the inoculum, because low density gives insufficient biomass and high density produces too much biomass resulting in depletion of nutrients necessary for protease fermentation. Different inoculum sizes were used and 3% (v/v) of 24 h old *Bacillus subtilis* ML-4 gave the best result, producing 204.81 U/mL of protease (Fig. 2). According

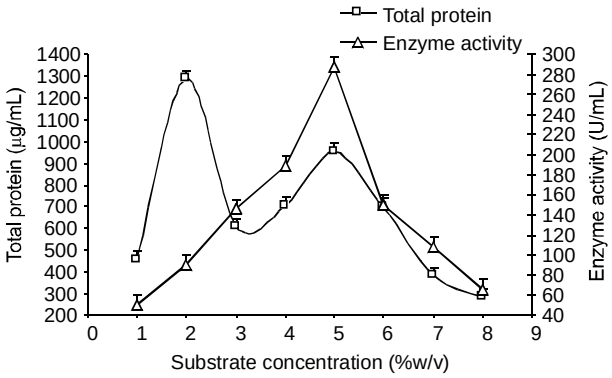

Fig. 1. Effect of substrate concentration on the alkaline protease by *Bacillus subtilis* ML-4.

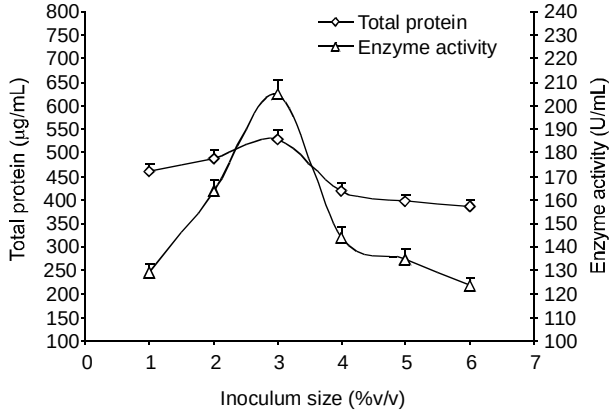

Fig. 2. Effect of inoculum size on the production of alkaline protease by *Bacillus subtilis* ML-4.

to Hornbaek *et al.* (2004) and Mangat and Mandahr *et al*. (1998), inoculum size has crucial role in the fermentation process. Tunga *et al*. (1998) stated that the inoculum has some optimum value for fermentation which depends on the microbial species. EI-Safey and Abdul Raouf (2004) optimized 24 h inoculum and size 1.0 cell/mL (7.0×10^3) for the production of protease by *Bacillus subtilis*.

Effect of initial pH. The metabolic activities of the micro-organism were very sensitive to pH variation. The maximum protease activity (205.54 U/mL) was found at pH 9 by *Bacillus subtilis* ML-4, however, a further change in pH decreased the enzyme yield. Ali and Roushdy (1998) also reported that optimum pH has important role in enzyme production (Fig. 3). The microorganisms exhibit more than one pH optimum for growth depending on the growth conditions, particularly, metal ions and temperature (Arai *et al*., 2003). The maximum protease yield was observed by Ul-Haq and Mukhtar (2006) at pH 9 for *B. subtilis* IH-72 in a bioreactor. Johnvesly *et al*. (2002) documented high level of extra cellular thermostable protease activity by thermoalkaliphilic *Bacillus* sp. JB-99 at pH 11.

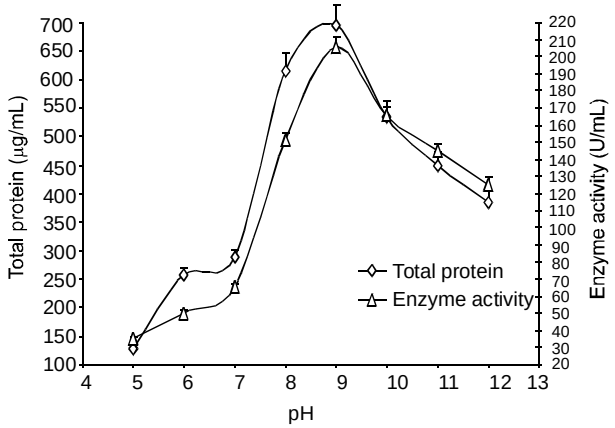

Fig. 3. Effect of pH on the production of alkaline protease by *Bacillus subtilis* ML-4 at 37 °C for 48 h. Bars represent S.D.

Effect of temperature on protease production. Maximum units of protease 283.89 U/ mL (Fig. 4) were obtained at 37 °C. Banerjee *et al*. (1999), working with *Bacillus brevis* in shake flask, also observed maximum activity of the enzyme at 37 °C. Joo *et al*. (2003) optimized 45 °C temperature for protease working with the *Bacillus horikoshii*. The thermoalkaliphilic *Bacillus* sp. produced thermo stable protease at 70 °C in rotary incubator shaker (Johnvesly *et al*., 2002). In addition to that, the optimum temperature for protease production was between 30 and 45 °C. Jobin and Grenier (2003) and Wery *et al*. (2003) investigated the production of proteases by *Streptococcus suis* serotype 2 and recorded optimum production of protease in the temperature range of 25 to 42 °C. However, proteinase production was not affected by the temperature in the range of 7-45 °C (Garcia de Fernando *et al*., 1991). These variations in incubation temperature might be due to difference in nature as well as type of various microbial specie.

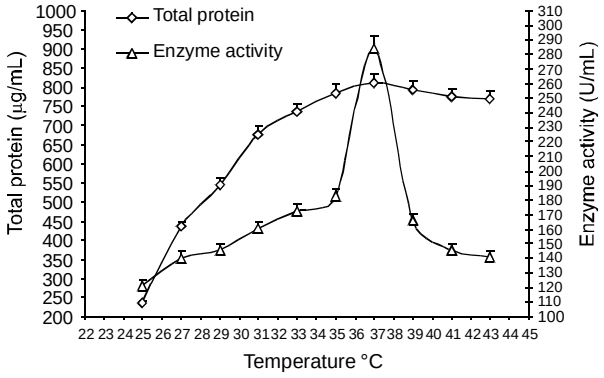

Fig. 4. Effect of temperature on the production of alkaline protease by *Bacillus subtilis* ML-4 at 9 pH for 48 h. Bars represent S.D.

Stability study. ***Effect of metal ions on the thermostability*.** The thermo-stability of alkaline protease was examined by measuring the residual activity at 40 °C after incubation of the enzyme without substrate at various temperatures ranging from 30 to 70 °C in the presence of Ca^{2+},Cu^{2+} and Mg^{2+} ions and with substrate for 30 min at 40 °C (Fig.5). The enzyme was found stable up to 50 °C; above this temperature, its activity decreased. The enzyme showed its 100% activity

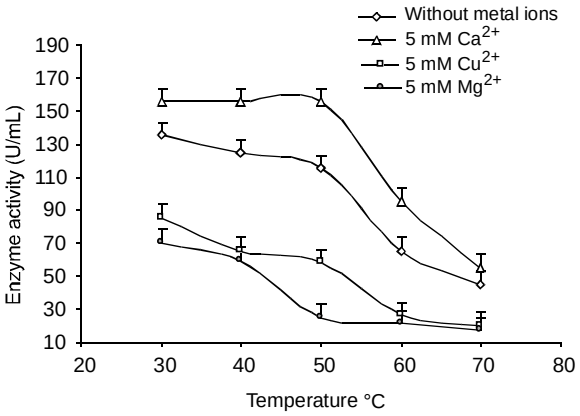

Fig. 5. Effect of metal ions on the thermostability of alkaline protease produced by *Bacillus subtilis* ML-4. Bars represent S.D.

at 50 °C and 51% at 60 °C in the presence of metal ions. Johnvesly *et al*. (2002) observed that 70 °C was the optimum temperature for protease activity of thermoalkaliphilic *Bacillus* sp. JB-99. Lee *et al*. (2002) reported that the optimum temperature for protease production ranged from 40 to 50 ºC. On the other hand, the highest activity of extracellular alkaline protease produced by the alkalophilic bacterium *Alcaligenes faecalis* was exhibited at 55 °C (Thangram and Rajkumar, 2002). Ammar *et al.* (2003) reported the optimum temperature for thermostable purified protease enzyme to be 55 °C. Nadeem *et al.* (2008), reported that 5 mM Ca^{2+} increased the stability of alkaline protease.

***Effect of pH on the stability*.** pH stability studies showed the enzyme to be stable at pH 10 but lost 50% of its residual activity at pH 11 (Fig. 6). pH level is one of the factors affecting the structure of not only enzymes but all proteins. pH values beyond the range of 8-11 could alter three-dimensional structure of alkaline protease by disturbing the electrostatic interactions among the charged amino acids, resulting in loss of enzyme activity. Similar results were reported by Sookkheo *et al*. (2000), who found 60% proteolytic retention at pH 10 in the presence of 5 mM Ca^{2+} ions. Optimal pH for purified extracellular alkaline protease produced by the alkalophilic bacterium *Alcaligenes faecalis* was 9.0. Thangram and Rajkumar (2002) and Lee *et al*. (2002) reported that the optimum pH of purified protease was 8. The enzyme was stable at pH 5.0-12 (Sun *et al*., 1997). Nadeem *et al*. (2008), reported that 5 mM Ca^{2+} increased the stability of alkaline protease.

The results of stability studies showed that alkaline protease produced by *Bacillus subtilis* ML-4 was stable over the range of temperature 30-60 °C and pH 8 to 11 in the presence of metal ions. The properties indicated that the enzyme can be used as potential ingredients in detergent formulation.

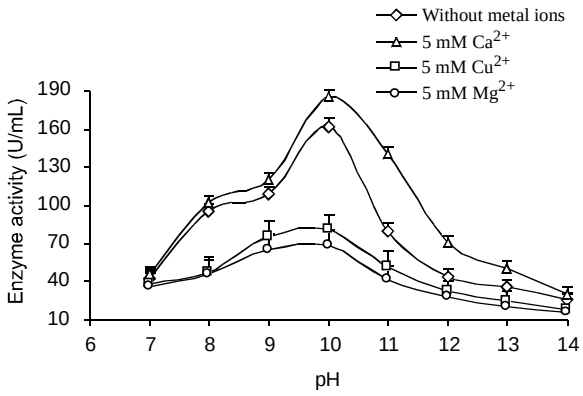

Fig. 6. Effect of metal ions on the pH stability of alkaline protease produced by *Bacillus subtilis* ML-4. Bars represent S.D.

Conclusion

The present study was conducted to optimize the parameters for maximum production of alkaline protease by *Bacillus subtilis* ML-4 grown on the medium containing poultry feed waste (5%), K_2HPO_4 (0.3%) $CaCl_2$ (0.03%) and $MgSO_4$ (0.015%). Maximum enzyme production (246.25±1.86U/ml) was obtained with 5% substrate concentration at initial pH 9 and 3% inoculum size after 48 hr of incubation at 37 °C. The enzyme was stable at temperature 30-60 °C and pH 8-11 in the presence of metal ions.

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