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Optimization and production of proteinacious chicken feather fertilizer by proteolytic activity of *Bacillus* sp. MPTK 6

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Abstract

This study illustrated that optimization of inorganic nutrients, metal ions and various cost-effective substrates yield a higher amount of solubilised protein which can be recovered from chicken feather powder by proteolytic activity of *Bacillus* sp. MPTK6. The test organism used in this study was isolated from feather composting soil in keratin enrichment medium at pH 8.5. The isolate *Bacillus* sp. MPTK6 showed a clear zone on the keratin enriched medium. Magnesium sulphate at 0.5% yielded high protease production and solubilisation where as KH_2PO_4 and K_2HPO_4 exhibited the same effect at 0.3%. Zinc sulphate and ferrous sulphate (15 µg/ml) significantly stimulated the enzyme production and protein yield. Corn steep liquor (0.3%) and peptone (0.3%) exert a very strong stimulatory effect on protease yield as well as protein solubilisation.

Keywords: Solubilised protein; *Bacillus* sp.; Magnesium sulphate; Zinc sulphate; Ferrous sulphate.

Introduction

Feathers are produced in large amounts as a waste by-product at poultry-processing plants, reaching millions of tons annually throughout the world (Manczinger *et al*., 2003; Fakhfakh *et al*., 2010). Waste products from food industries having proteinacious materials have invited researchers to explore new non-conventional resources of protein. Attempts have been made to use waste material of food processing industries like agriculture1, poultry, meat and fish industries for recovery of protein (Zelinik *et al*., 1999; Kawasaki and Glenn, 1999). Annually over 100 million tons of fish are harvested worldwide, and about half of the total catch is discarded as processing waste (You *et al*., 2000). Waste management seems to be the only possible option answering both the needs. Recently different research groups around the world have given lot of emphasis by finding ways to recover proteins out of waste products in Poultry industry (Moritz and Latshaw , 2001; Odetallah *et al*., 2003).

Feathers, consisted mainly of keratin characterized by its high recalcitrant nature, could be an important protein source in animal feedstuff. The protein shortage for food and feed leads us to look for a new protein sources from wastage products like feather wastes (Odetallah, 2003). Feathers are a significant source of protein for livestock because of their high protein content (>85%), (MacAlpine and Payne, 1977; Macedo *et al*., 2005; Thys and Brandelli, 2006; Anbu *et al*., 2007; Riffel *et al*., 2007; Kumar *et al*., 2008). Feathers contain large amounts of cystine, glycine, arginine, and phenylalanine (Onifade, 1998).

Alkaline proteases are among the most important industrial enzymes; they are primarily employed as detergent additives, accounting for 40–60% of the global enzyme market. Alkaline proteases are group of proteolytic enzymes that are able to hydrolyze insoluble hard proteins more effectively than other proteases such as trypsin, pepsin and papain (Onifade, 1998; Papadopoulos, 1986; Gradisar *et al.*, 2000). The genus *Bacillus* provides most of the alkaline proteases with

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commercial value, offering the advantages of being easy to culture and maintain and they are potentially valuable in the bioconversion of keratinous wastes; in the detergent, fertilizer, biopolymer, pharmaceutical, and animal feed industries; and in leather processing and hydrolysis of prion proteins as well (Riffel and Brandelli, 2006). Therefore, such microorganisms and enzymes have been the focus in several studies (Macedo *et al*., 2005; Thys and Brandelli, 2006; Anbu *et al*., 2007; Riffel *et al*., 2007) and *Bacillus* alkaline proteases are reported to be among the most efficient keratin degraders (Kumar *et al*., 2008).

Considering the above facts this study was aimed to optimize the inorganic nutrients, metal ions and various cost-effective substrates for a higher yield of solubilised protein that can be recovered from chicken feather powder by proteolytic activity of *Bacillus* sp. MPTK6.

Materials and methods

Feathers processing

Chicken feathers (CF), supplied by a local poultry industry were washed threefold with tap water and finally with distilled water. The washed feathers were dried at 90°C for 22 h and then stored at room temperature prior to microbial treatment (Fakhfakh *et al*., 2010).

Isolation and screening of keratinolytic bacteria

The test organisms used in this study were isolated from feather composting soil near local poultry farm in Chennai, India. 1 g of soil sample was added to the keratin enrichment medium which contains (%): NH_4Cl 0.05, NaCl 0.05, K_2HPO_4 0.03, KH_2PO_4 0.04, $MgCl_2.6H_2O$ 0.024, yeast extract 0.01, and 1% feather keratin substrate 1 at pH 8.5 for 3 d under shaken condition. The enriched sample (1 ml) was suspended in 9 ml of sterilized water and was spread on the same feather enriched medium by addition of 1.7% agar and incubated at 37°C for 72 h. After incubation the plates ware stained with Coomassie blue for the observation of clear zones around the margins of colonies and were picked up as alkaline keratinase producers.

Optimization of media components

Surface culture fermentation was carried out using 150 ml conical flask, which contained 30 ml fermentation medium (FM) consisting of: glucose, 0.05; urea, 0.025; $MgSO_4.7H_2O$, 0.01; and KH_2PO_4, 0.01 g/ml; chicken feather powder (CFP) 2.5 g; and pH, 4.0. Inoculated medium were incubated at 28ºC (± 0.5ºC in BOD incubator) for 14 d. Sterile solution of $MgSO_4.7H_2O$, K_2HPO_4, KH_2PO_4, NaCl, KCl and $CaCl_2$ added individually to the sterile medium keeping all the factors constant except one, effects of which was under investigation. Sterile solutions of trace metals were added separately with glucose, urea, $MgSO_4.7H_2O$ and K_2HPO_4 solutions. Chemicals were purified from trace elements by the method of chloroform extraction (Majumder and Bose, 1960; Pratiti and Banik, 1998). In this method, the required amount of aqueous solution of each was mixed well with 0.1 g of 8-hydroxyquinone and chloroform in a separating funnel, first at pH 5.2, then at pH 7.2. The chloroform-dissolved impurities were extracted out. The process was repeated at two different pH. The solutions were made chloroform free by heating in a water bath and sterilized separately. Complex nutrients were added as sterile solution based on their solid content (Basu and Banik, 2005).

Protease assay

The protease activity was measured by the method described by Meyers and Ahearn (1977). One milliliter of culture filtrate was added with 1ml of 1% (w/v) casein solution in glycine–NaOH buffer of pH 10.5 and incubated for 10 min at 60°C. The reaction was stopped by addition of 4 ml of 5% trichloroacetic acid. The reaction mixture was centrifuged at 3000×g for 10 min and to 1 ml of the supernatant 5 ml of 0.4 M Na_2CO_3 was added followed by 0.5 ml Folins Ciocalteus reagent. The amount of tyrosine releases was determined spectrophotometrically by taking optical density at 660 nm against the enzyme blank.

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One unit of protease activity was equivalent to amount of enzyme required to release 1g of tyrosine/ml/min under standard assay conditions.

Total protein content

The amount of protein was estimated by Bradford method (1976) using Bovine Serum Albumin (BSA) as a standard according to the instruction manual of Quick Start Bradford Protein Assay.

Biomass determination

Bacteria biomass was determined by measuring the absorbance at 600nm (Henroette *et al.,* 1993).

Determination of nitrogen content of CFP

Nitrogen content of CFP was measured before and after fermentation by modified micro-kjeldahl digestion method (Meyers and Ahearn, 1977). The percentage of solubilised nitrogen was calculated comparing the nitrogen value of degraded and non-degraded CFP. Protein content of CFP was obtained by multiplying nitrogen content with the factor 6.25.

Results and discussion

Poultry feather constitutes the most abundant keratinous material in nature. Their accumulation results from the poultry processing industry. Thus this waste product carries potent ecological implications, especially with burgeoning global poultry production. Recycling of feathers is a subject of interest among animal nutritionists, because of its potential as a cheap and alternative protein feedstuff (Fakhfakh *et al*., 2010). This study investigated the effect of various cost-effective nutrients, trace metals and essential inorganic nutrients on keratinolytic *Bacillus* sp. MPTK6 for protease yield and production of soluble protein from chicken feather powder (CFP). The test organism used in this study was isolated from feather composting soil in keratin enrichment medium at pH 8.5. The isolate *Bacillus* sp. MPTK6 showed a clear zone on the keratin enriched medium and hence, this isolate was selected for further studies.

Table .1 Effect of $MgSO_4.7H_2O$ on protease activity and solubility of CFP

	0.25%	0.50%	0.75%	1%	1.25%
Solubility (%)	35	41	37	35	32
Mycelial weight (g/100 ml)	2.8	3	3	3.1	3.2
Soluble protein (g/l)	4.9	9.7	7.7	6.8	5
Protease activity (U/ml/min.)	900	1100	955	900	935

Table 2. Effect of NaCl on protease activity and solubility of CFP

	0.02%	0.05%	0.075%	0.1%
Solubility (%)	45	39	34	32
Mycelial weight (g/100 ml)	3.5	3.3	3	3
Protease activity (U/ml/min.)	1400	1100	950	900

Table 3. Effect of KCl on protease activity and solubility of CFP

	0.02%	0.05%	0.075%	0.1%
Solubility (%)	43	37	39	35
Mycelial weight (g/100 ml)	3.1	3	3.3	3
Protease activity (U/ml/min.)	1200	1000	980	850

Growth of *Bacillus* sp. MPTK6 was proportional to Mg content of the medium, which was present as sulphate. Different concentrations of $MgSO_4.7H_2O$ (0.25, 0.5, 0.75, 1 and 1.25%) were tested and 0.75% gave highest protease production and solubilisation (Table 1). Mg^{2+} functions by influencing enzyme system and protease is one of them (Mukhopadhyay and Banik, 1991; Bilgrami and Verma, 1994).

Table 4. Effect of $CaCl_2$ on protease activity and solubility of CFP

	0.02%	0.05%	0.075%	0.1%
Solubility (%)	42	37	34	32
Mycelial weight (g/100 ml)	3	3.1	3	3
Protease activity (U/ml/min.)	1300	1450	1100	950

Phosphate source was provided by potassium phosphate which was responsible for buffering the medium (Ghosh and Banik, 1998). Two different inorganic phosphate sources were added to medium in different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5%). Phosphate has been reported to be optimum for protease production at a concentration of 0.2 to 0.3% (Ellaiah *et al*., 2002). It was found that 0.3% was optimum and amount in excess of 0.4% showed an inhibition on cell growth and repression in protease production (Table 2 &Fig.1 and 2).

Different concentrations of NaCl, KCl and $CaCl_2$ (0.02, 0.05, 0.075 and 0.1%) were added to fermentation medium. There was a strong inhibitory effect of NaCl at 0.1% on protease

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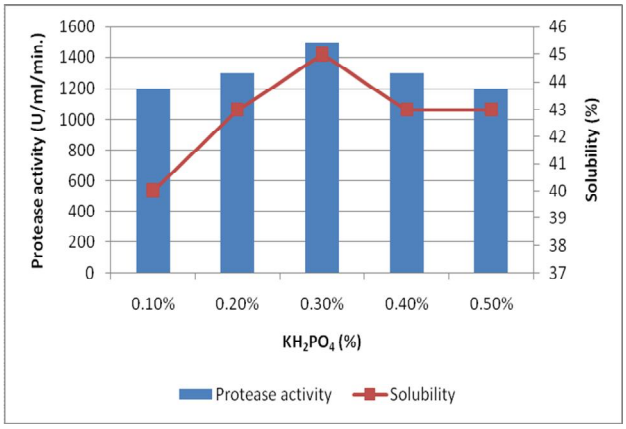

Fig. 1. Effect of KH_2PO_4 on protease activity and solubility of CFP

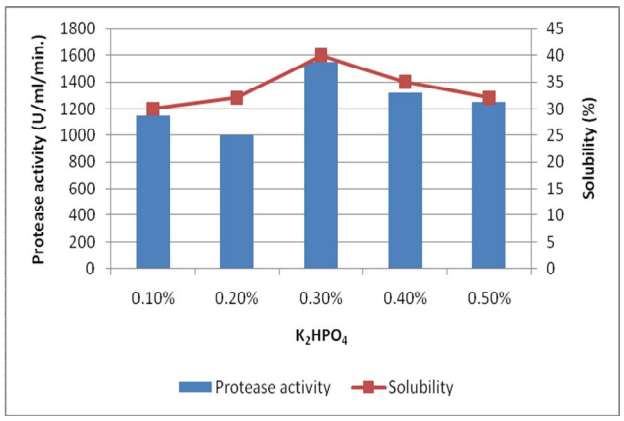

Fig. 2. Effect of K_2HPO_4 on protease activity and solubility of CFP

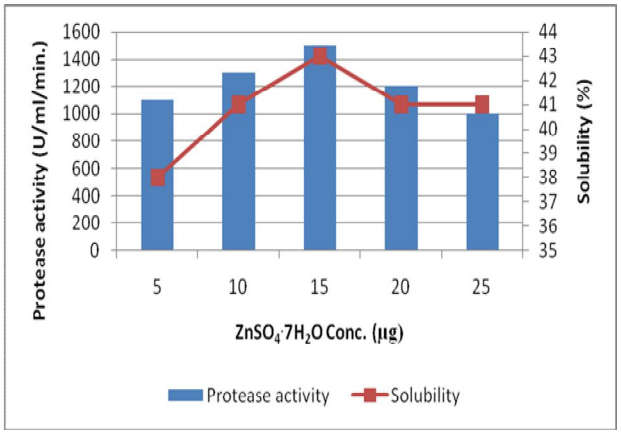

Fig. 3. Effect of $ZnSO_4.7H_2O$ on protease activity and solubility of CFP

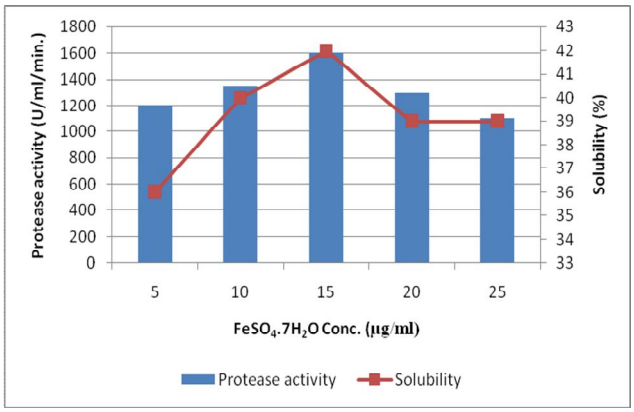

Fig. 4. Effect of $FeSO_4.7H_2O$ on protease activity and solubility of CFP

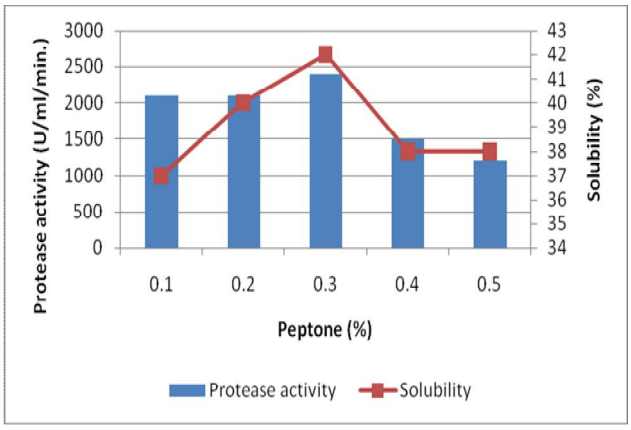

Fig. 5. Effect of peptone on protease activity and solubility of CFP

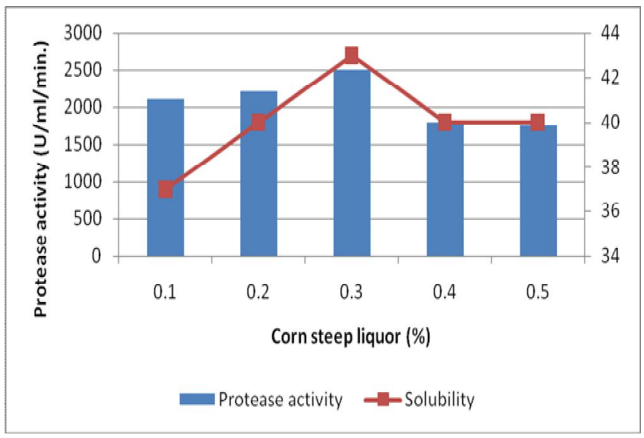

Fig. 6. Effect of corn steep liquor on protease activity and solubility of CFP

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production, protein solubilisation and cell growth (Table 2). KCl (0.02%) has a stimulatory effect on total solubilisation process (Table 3). $CaCl_2$ (0.05%) has slight positive effect on enzyme production; however, higher concentration imparts a negative effect (Table 4). Results of this study with NaCl were in agreement with the findings of previous studies (Bilgrami and Verma, 1994) where sodium salts like NaCl, Na_2SO_4 and Na_2S depressed proteolytic activity of microorganisms. Growth impairment of *Bacillus* sp. MPTK6 with KCl (< 0.075%) was observed in this study whereas Ca^{2+} resulted in partial inhibition. The results are in accordance with the findings of Basu and Banik (2005).

Trace metals were tested at different concentrations (5, 10, 15, 20 and 25 µg/ml). Zinc sulphate and ferrous sulphate (15 µg/ml) significantly stimulated the enzyme production and protein yield (Fig.3). Divalent metal ions (calcium, copper, cobalt, boron, iron, manganese, molybdenum) are required in fermentation medium for optimum production of protease (Ellaiah *et al*., 2002). Iron, being an integral part of the protoplasm, is associated with various enzyme systems. It was found that Fe^{3+} had a stimulatory effect on the enzyme production. It was found that Zn^{2+} (>15 µg/ml) had inhibitory effect on the enzyme production (Fig.4). The finding of this study was similar in case of Mukhopadhyay and Banik (1991).

The culture environment has a dramatic influence on enzyme production especially Carbon and nitrogen sources play a crucial role in enzyme induction in bacteria (Elibol and Ozer, 2001). Complex nutrients [peptone, extracts of yeast, wheat–bran, rice-bran, beef and malt, paddysoak-liquor, corn-steep-liquor (CSL)] were tested in a concentration of 0.1, 0.2, 0.3, 0.4 and 0.5% based on their solid content. Peptone (0.3%) exerts a very strong stimulatory effect on protease yield as well as protein solubilisation (Fig.5). CSL (0.3%) was found to have maximum stimulatory effect on protease production (Fig.6). Results from present study are supported by previous reports where CSL was suitable nitrogen source for alkaline protease production and used as a cheap source of nitrogen (Malathi and Chakrabarty, 1991; Sen and Satyanarayana, 1993; George *et al*., 1997; Basu and Banik, 2005).

Conclusion

Poultry feather and other keratins are potential protein feed resources for livestock. The natural existence of keratin-degrading microorganisms offers a feasible microbial enzyme technology capable of producing a more nutritionally balanced and digestible product than does the conventional hydrothermal processing. Furthermore, the microbial enzyme technology is low-energy-consuming and environmentally friendly. To conclude, this study illustrated that optimization of inorganic nutrients, metal ions and various cost-effective substrates yield a higher amount of solubilised protein which can be recovered from chicken feather powder by proteolytic activity of *Bacillus* sp. MPTK6 and used as an animal feed in future after careful investigations.

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