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Optimization of alkaline protease production and its fibrinolytic activity from the bacterium *Pseudomonas fluorescens* isolated from fish waste discharged soil

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Pseudomonas fluorescens **AU_{17} was isolated from the fish waste discharged soil and it was tested for its ability to produce the protease enzyme. The effect of different production parameters such as temperature, pH, carbon and nitrogen sources and sodium chloride concentration for protease production by the isolated bacterial strain were studied. The optimum conditions observed for protease production were temperature (37°C) and pH 9, 1% wheat bran for carbon source, 0.5% peptone for nitrogen source; magnesium sulphate has less inhibitory effect among the metal ions tested. Under optimized parameters, maximum protease activity was 0.9343 U/ml/min. The bacterial isolate has potential that could be commercially exploited to assist in protein degradation in various industrial processes.**

Key words: Alkaline protease, casein agar, fish contaminated soil, *Pseudomonas fluorescens,* fibrinolytic activity.

INTRODUCTION

Pseudomonas fluorescens is well known as a major psychrotrophic contaminant of raw milk stored in refrigerated tanks (Law et al., 1977). *P. fluorescens* is a ubiquitous soil microorganism that inhabits the surfaces of seeds and roots. Some strains of *P. fluorescens*, when growing in association with plants, can protect them from infection by plant pathogens (Thomashow and Weller, 1995). One such strain, *P. fluorescens* Pf-5, produces a number of antibiotics, including pyoluteorin (Plt) (Howell and Stipanovic, 1980), pyrrolnitrin (Prn) (Howell and Stipanovic, 1979), and 2,4-diacetylphloroglucinol (Phl) (Nowak-Thompson et al., 1994). Of the three antibiotics, Plt is most toxic to the oomycete *Pythium ultimum* (Maurhofer et al., 1994), which can infect seeds and roots of many plant hosts and cause seedling death and root rot (Martin and Loper, 1999).

Proteases or proteinases are proteolytic enzymes which catalyze the hydrolysis of proteins. Based on their structures or properties of the active site, there are several kinds of proteases such as metallo, serine, acidic, carboxyl, alkaline and neutral proteases. Proteases are industrially important enzymes constituting a quarter of the total global enzyme production (Kalaiarasi and Sunitha, 2009). Proteases are industrially important due

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to their wide applications in leather processing, detergent industry, food industries, pharmaceutical, textile industry etc., (Jellouli et al., 2009; Deng et al., 2010). The Industrially important proteases are obtained from plants, animal organs and microorganisms, with the majority obtained from microbial sources. Microorganisms are the most important sources for enzyme production. For manufacture of enzymes for industrial use, isolation and characterization of new promising strains using low-priced carbon and nitrogen source is a continuous process. Habitats that contain protein are the best sources to isolate the proteolytic microorganism (Dalev, 1994). Waste products of meat, poultry and fish processing industries can supply a large amount of protein rich material for bioconversion to recoverable products (Gaustevora et al., 2005). This present study aims to isolate protease-producing bacteria from fish shop waste discharged soil. The optimization of the extracellular protease production is influenced by several physical parameters. This study presents effect of different cultural conditions on production of protease from *P. fluorescens* isolated from fish discharged soil.

MATERIALS AND METHODS

Screening and isolation of proteolytic bacteria

Fish waste discharged soils were collected from Chidambaram fish market. One gram of the contaminated soil sample was weighed aseptically into 100 ml of sterile distilled water, agitated for 45 min on a shaker. 0.2 ml was cultured in 1% casein with nutrient agar plates and incubated at 37°C for 24 h. A total of 23 bacterial isolates from enriched sample was plated over nutrient agar medium containing 0.4% gelatin (Harrigan and Cance, 1966). After 24 h of incubation, plates were flooded with 1% tannic acid. Isolates having a higher ratio of clearing zone to colony size were grown in liquid broth and the amount of protease production was determined from culture filtrate. The isolate which showed higher protease activity was selected and maintained on nutrient agar slants selected isolate was identified based on morphological and biochemical characteristics following the method described by Kannan (2002).

Production of protease enzyme

Yeast extract casein medium was the excellent medium for the production of protease enzyme. In this basal medium contains, glucose 10 g, casein 5 g, yeast extract 5 g, KH_2PO_4 2 g, Na_2CO_3 10 g (Naidu and Devi, 2005). The test bacteria were inoculated into yeast extract casein medium and incubated at 37ºC overnight in shaking incubator. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm at 4°C for 15 min to obtain the crude extract, which served as enzyme source.

Protease assay

The protease activity was estimated by the method described by Beg et al. (2003). Following incubation, the bacterial broth was centrifuged at 5000 rpm for 20 min at 4°C to obtain the cell free supernatant (CFS). 1 mL of CFS was added to 1 mL 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 the addition of 4 mL of 5% trichloroacetic acid. The reaction mixture was centrifuged at 3000 rpm 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-Ciocalteu reagent. The amount of tyrosine released was determined using a UV-VIS spectrophotometer (SANYO Gallenkamp, Germany) at 660 nm against the enzyme blank. One unit of protease activity was defined as the amount of enzyme required to release 1 µg of tyrosine per mL per min under standard assay conditions.

Process optimization for maximum protease production

Effect of Inoculum concentration

The concentration effect of the inoculums on protease production was determined by inoculating the production medium with different concentration ranging from 2 to 7% of overnight grown selected bacterial culture. The inoculated medium was incubated for 24 h. The culture medium was centrifuged at 5000 rpm at 4°C for 15 min. Protease activity was determined in the cell free supernatant.

Effect of Incubation time

The incubation time effect on protease production was determined by incubating the culture medium at different time intervals (24 - 168 h) with an interval of 24 h.

Effect of pH

The pH effect of the protease production was carried out by adjusting the pH of the production medium in the range of 5 to 11 using 1 N HCl and 1 N NaOH as found appropriate. After the incubation time, the culture medium was centrifuged at 5000 rpm for 15 min in a refrigerated centrifuge at 4°C. Protease activity was determined in the supernatant.

Effect of temperature

The yeast extract casein medium at pH 9 was inoculated with 2% overnight grown selected bacterial strain. The broth was incubated at different temperatures ranging from 27 - 67°C at 10°C interval for 24 h. Protease activity was determined after 24 h.

Effect of carbon sources

The effect of various carbon sources such as starch, wheat bran, rice bran, maltose and sucrose at a concentration of 1% was examined by replacing glucose in the production medium.

Effect of nitrogen sources

Various nitrogen sources like beef extract, tryptone, glycine, peptone and casein were examined for their effect on protease production by replacing 0.5% of yeast extract in the production medium.

Effect of metal ions

Influence of various metal ions on protease production was determined by incubating the yeast extract casein medium with different metal ions such as $BaCl_2$, $CaCl_2$, $MgSO_4$, K_2HPO_4 and $CuSO_4$ at a con-

Figure 1. Protease production of *P. fluorescens* in Skim Milk Agar.

centration of 0.2%. All the experiments were carried out in triplicates and results presented are the mean of three values.

Determination of fibrinolytic activity

Four pieces of cotton fabric were individually impregnated with 500 µL of blood and the blood stains were allowed to dry. Then the fabrics were soaked in 2% (v/v) formaldehyde for 30 min and rinsed with water to remove excess formaldehyde (Adinarayana et al., 2003). Upon drying, the fabric pieces were separately incubated with 1 mL of the partially purified protease, 1 mL of the partially purified protease with detergent, 1 mL of sterile distilled water with detergent and 1 mL of sterile distilled water at 37°C for 1 h. Following incubation, the fabric pieces were rinsed with water, dried and checked for the extent of blood removal.

Statistical analysis

Data obtained were analyzed by statistical method described by Steel et al. (1997). MS Excel software was used to draw graphs.

RESULTS

Isolation and screening of proteolytic bacteria

A total of 23 bacteria were isolated from the dairy sludge examined. When tested for their proteolytic potential, 8 isolates (AU_5, AU_9, AU_{11}, AU_{14}, AU_{17}, AU_{19}, AU_{21}, and AU_{23}) demonstrated clear zones around the streak on the skimmed milk agar (an indication of protease production) (Figure 1). Among these isolates, AU_{17} demonstrated the highest zone of proteolysis (24 mm) as compared to the other isolates and therefore it was selected for further studies (Table 1).

Table1. Protease activity of isolated bacteria.

Bacterial colonies	Protease activity (U/ml/min)
AU_5	0.3245
AU_9	0.1798
AU_{11}	0.5709
AU_{14}	0.4212
AU_{17}	0.9343
AU_{19}	0.2312
AU_{21}	0.3112
AU_{23}	0.1699

Identification of the selected bacterial isolate

Based on the morphological characters, the isolate AU_{17} was found to be gram negative short rod showing motility. Biochemical characterization revealed the identity of the isolate as *P. fluorescens*. The morphological and biochemical characterization are presented in Table 2.

Process optimization for maximum protease production

The protease production was carried out in 1-7% inoculums and the results are 0.1-0.25 respectively. In 2%, inoculums showed the higher activity of protease production likes 0.25%. The enzyme activity was gradually decreased in 3-7% (Figure 2).

The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Maximum protease production was achieved at pH 9.0 by *P. fluorescens*. The production of protease enzyme increased as pH of the medium increases and reaches maximum at pH 9.0. After pH 9.0, there was a decrease in enzyme production (Figure 3).

P. fluorescens was capable of producing protease in the range of 7 - 67°C with production maximum at 37°C. However, increase in temperature beyond 37°C led to decline in production of enzyme proving that temperature plays a major role in protease production (Figure 4).

Protease production was found to be maximum at 24 h. The enzyme activity gradually decreased from 24 to 168 h. This finding is in partial agreement with findings of Kumar et al. (2002) who reported that *Pseudomonas* sp. S22 showed a peak for protease production at 24 h of incubation and again peaks at 108 h which contradicts the finding of this present study (Figure 5).

The addition of carbon source in the form of either monosaccharide or polysaccharides could influence the production of enzyme (Sudharshan et al., 2007). Among the carbon sources, wheat bran and maltose were found to support protease production. The isolated strain showed high enzyme yield (0.389 U/ml/min), when wheat bran was used as carbon source (Figure 6).

Table 2. Biochemical tests of the selected bacterium.

Biochemical character	Bacterial colony AU_{17}
Gram staining	Gram negative
Motility test	Motile
Indole production test	Negative
Methyl red test	Negative
Voges Proskaurer test	Negative
Citrate utilization test	Positive
Catalase test	Positive
Oxidase test	Positive
Urea hydrolysis	Positive
Nitrate reduction	Positive
Starch hydrolysis	Negative
Gelatin hydrolysis	Positive
Fluorescence on King's B medium	Positive
Growth at 4°C and 41°C	Good
Levan formation	Positive

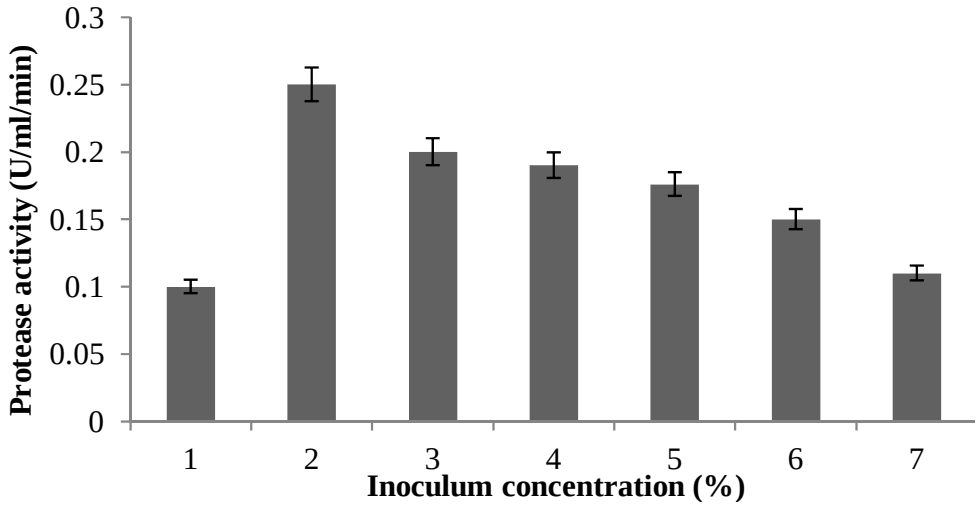

Figure 2. Effect of inoculum concentration on protease production by *Pseudomonas fluorescens.*

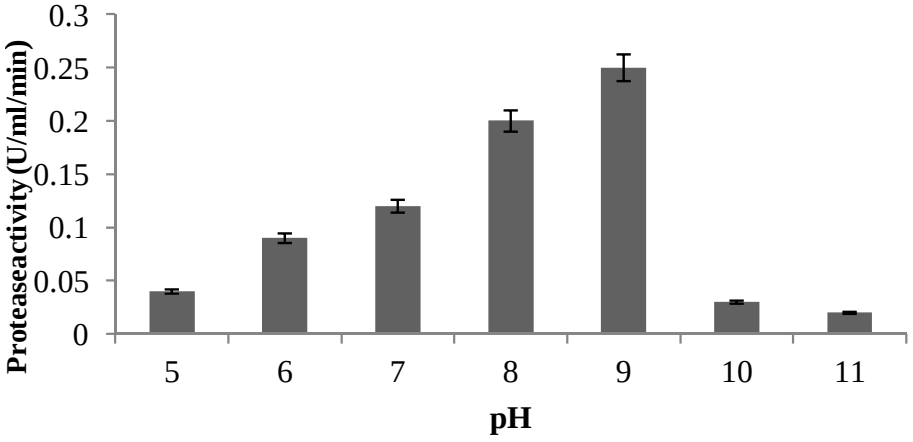

Figure 3. Effect of pH on protease production by *Pseudomonas fluorescens.*

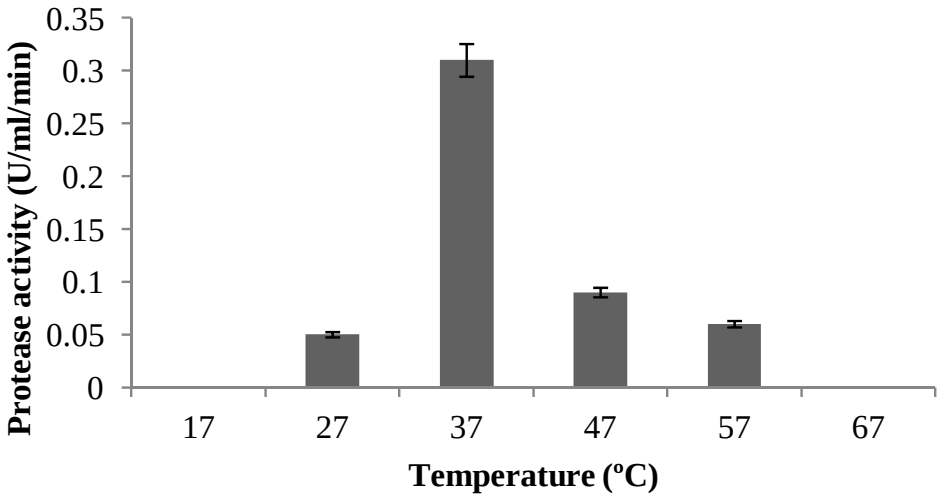

Figure 4. Effect of temperature on protease production by *P. fluorescens.*

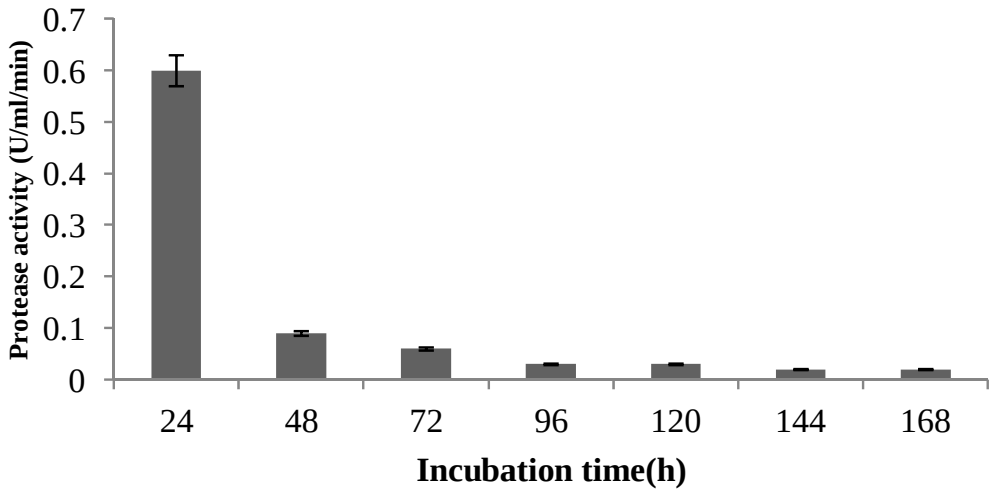

Figure 5. Effect of incubation time on protease production by *P. fluorescens.*

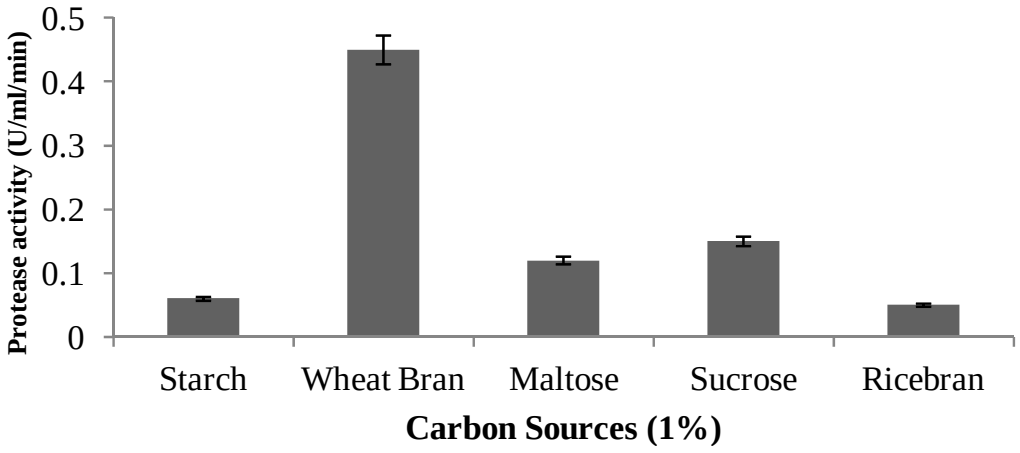

Figure 6. Effect of carbon source on protease production by *P. fluorescens.*

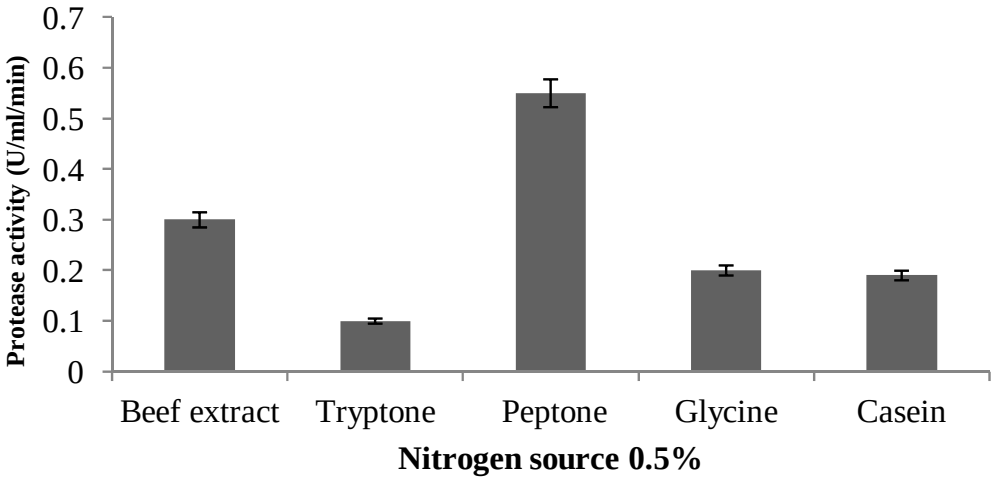

Figure 7. Effect of nitrogen for protease production by *P. fluorescens.*

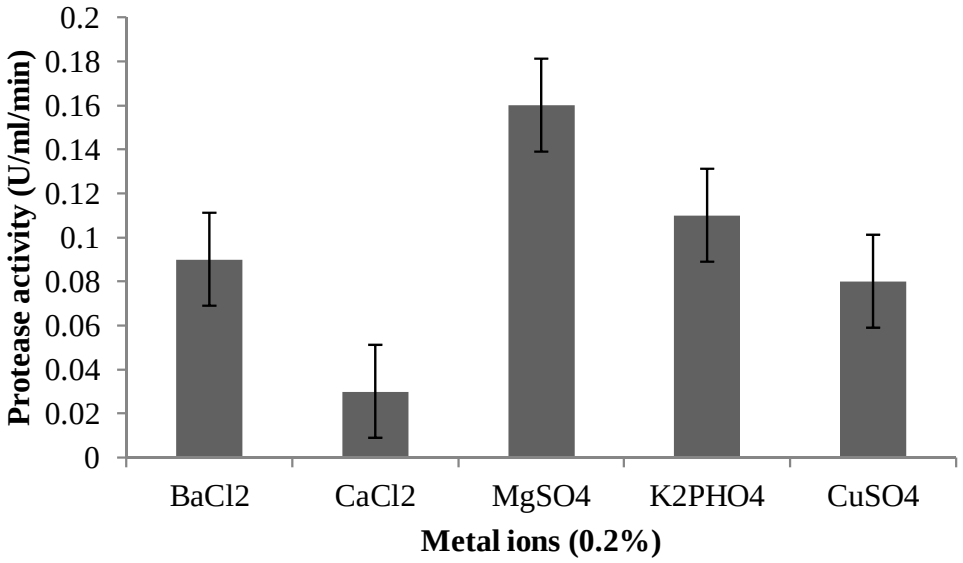

Figure 8. Impact of metal ions on protease production by *P. fluorescens.*

Nitrogen is metabolized to produce primarily amino acid, nucleic acid and protein and cell wall components. These nitrogen sources have regulatory effect on the enzyme synthesis. Production of protease is highly dependent on both carbon and nitrogen sources available in the medium (Shanthakumari et al., 2010). Testing the effect of various nitrogen sources on protease production, it was found that peptone gave the highest enzyme activity of 0.55 U/ml/min while. The inorganic nitrogen sources appeared to be less preferred when compared to the organic nitrogen sources in protease production observed after 48 hours of incubation (Figure 7).

A metal ion in media is an important factor that affects enzyme production. $CaCl_2$ at a concentration of 0.2% inhibits protease production followed by $CuSO_4$ and $BaCl_2$. Magnesium sulphate has less inhibitory effect on the production of protease. These results are in accordance with Wang et al., (2008), they reported that the optimized metal ion for protease production by *Chryseo* bacterium was 0.05% $MgSO_4$ (Figure 8).

Fibrinolytic potential of the protease

The degree of blood removal from the cotton fabric was found in the order of: partially purified protease with detergent > partially purified protease > sterile distilled water with detergent> sterile distilled water.

DISCUSSION

Proteases (EC 3.4.21-24 and 99; peptidyl-peptide hydrolases) are enzymes that hydrolyze proteins via the addition of water across peptide bonds and catalyze peptide synthesis in organic solvents and in solvents with low water content. In all living organisms, proteolytic enzymes are widely found and are essential for cell growth and differentiation (Vadlamani and Parcha, 2011). Maximum protease productions were achieved at 2% inoculum concentration. The enzyme activity gradually decreased from 3 to7%. Elibol et al. (2005) noticed the higher protease production in that 2.5% inoculum level.

The microbial cells are significantly affected by the environmental pH because they in fact have no mechanism for adjusting their pH. Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth (Bhattacharya et al., 2011). The pH of the culture strongly affects many enzymatic processes and transport compounds across the cell membrane. Maximum protease production was achieved at pH 9.0 by *P. fluorescens*. The production of protease increased as pH of the medium increases and reaches maximum at pH 9.0. After pH 9.0 there was a decrease in enzyme production. This suggests that there is a stimulation of enzyme production at alkaline pH. The results coincide with those of Kumar et al. (2002) which found out that protease production was maximum at pH 7 and 9 for *Bacillus* sp. strain S4 and *Pseudomonas* sp. strains S22 respectively. Similarly, Borriss (1987) found maximum alkaline protease production at pH 9 -13.

The highest enzyme yield was observed at pH 7.0 of the production of organic solvent protease by *P. aeruginosa* strain K. Neutral media increased the protease production as compared to acidic or alkaline media (Rahman et al., 2005). Likewise, when the protease production medium for *B. subtilis* was adjusted at different pH values with different buffers, results indicated that the best buffer was phosphate buffer and the optimum pH for production of protease was recorded at 7.0. A decline in the enzyme productivity occurred at both higher and lower pH values (Qadar et al., 2009). Certain *Bacillus* species produced protease over the entire range of pH investigated (pH 5 - 10). The optimum pH for maximum protease production from *Bacillus* SNR01 was at pH 7.0 (Josephine et al., 2012). Radha et al. (2011) studied the production and optimization of acid protease by *Aspergillus* sp. from soil. A gradual increase in protease at pH from 3.0 to 5.0 was reported (Radha et al., 2011), whereas, it declined at neutral and alkaline pH. Fungal acid proteases have an optimal pH range from 4 - 4.5 and they can be stable at pH values from 2.5 - 6.0. Maximum production of enzyme and fungal dry mass were observed at pH of 5. Similarly, Kumar et al. (2002) reported that the optimum pH was in the acidic range of 5.5 - 6.5 for acid protease production from solid tannery waste by Synergists species.

Temperature significantly regulates the synthesis and secretion of bacterial extracellular proteinase by changing the physical properties of the cell membrane (Balaji et al., 2012), therefore, temperature is a critical parameter that should be controlled in order to obtain an optimum proteinase production. In concurrence of the present study with previous findings, where the bacterial isolates like *P. aeruginosa* MTCC 7926, *Serratia liquefaciens* preferred 37°C for maximum production of protease (Patil and Chaudhari, 2011; Smita, 2012). The production of alkaline protease by *Bacillus halodurans* was investigated, wherein the maximal cell growth was seen at 50°C and maximum enzyme production was found at 37°C (Ibrahim and Al-Salamah, 2009). It is very essential to detect the optimum incubation time at which an organism exhibits highest enzyme activity since organisms show considerable variation at different incubation periods (Kumar et al., 2008). *P. fluorescens* was capable of producing protease in the range of 17 - 67°C inconsistent with what you have in the result however there was no production of enzyme at 17 and 67°C (Figure 3) so I suggest your range should be from 27- 57°C, with production of maximum at 37°C (Figure 3). However, increase in temperature beyond 37°C led to decline in enzyme production proving that temperature plays a major role in protease production. Fujiwara and Yamamoto (1987) also noticed that protease activity was high at 30°C for *Bacillus* sp.

The addition of carbon source in the form of either monosaccharide or polysaccharides could influence the production of enzyme (Sudharshan et al., 2007). Among the carbon sources, wheat bran and maltose were found to support protease production. The isolated strain showed high enzyme yield (0.389 U/ml/min), when wheat bran was used as carbon source (Figure 5). These results are in agreement with those of Naidu and Devi (2005) as wheat bran supported the maximum production of protease in *Bacillus* sp. Uyar and Baysal (2004) examined wheat bran and lentil husk, in that wheat bran showed highest protease production in *Bacillus* sp. Among the nitrogen sources, peptone produced maximum protease. Wang and Hsu (2005) found that casein and peptone were better nitrogen sources for protease production by *Prevotella ruminicolo* 23. However, production medium enriched with soybean meal has been reported as best nitrogen source for protease production as stated by Sinha and Satyanarayana (1999). A metal ion in media is an important factor that affects enzyme production. $CaCl_2$ at a concentration of 0.2% inhibits protease production followed by $CuSO_4$ and $BaCl_2$. Magnesium sulphate has less inhibitory effect on the production of protease. Wang et al. (2008) reported that the optimized metal ion for protease production by *Chryseo* bacterium was 0.05% $MgSO_4$.

It is very essential to detect the optimum incubation time at which an organism exhibits highest enzyme activity since organism show considerable variation at different

incubation periods (Kumar et al., 2012). This present study is in agreement with those of previous workers, who reported high proteolytic activity by *Bacillus* sp. Using beef extract for an incubation time of 48 h (Shivakumar, 2012). *P. aeruginosa* showed maximum protease activity at pH 9.5, temperature 37°C and 48 h of incubation time (Samanta et al., 2012). Protease production from *P. fluorescens* was found to be maximum at 24 h beyond which the enzyme activity gradually decreased from 48 to 168 h, which is in contrast to the present finding (Kalaiarasi and Sunitha. 2009). When applied alone, this protease removed blood stain from the fabric significantly.

The washing efficiency of the detergent was also remarkably increased with addition of the enzyme. Similar result of the usage of this enzyme as formulation in detergent preparation, was obtained earlier (Mala and Srividya, 2010).

The results of this present study elucidated that fish contaminated soil can be a very good source for isolating proteolytic bacteria. This study gains its importance since there is scarcity in terms of proteolytic enzymes from *P. fluorescens*. Considering the fact that enzyme production by microorganisms is under the influence of various growth conditions, the present investigation determined the optimum environmental and nutritional parameters for maximum production of protease from the isolate. The fibrinolytic nature of the enzyme alone and in synergy with the detergent evokes the idea that this particular organism may be exploited in the pharmaceutical and detergent industries in future.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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