

Optimization of Culture Conditions for the Production of An Extracellular Alkaline Protease from *Vibrio mimicus*

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Abstract. Wheat bran was used as the substrate for alkaline protease production by solid state fermentation. Among the isolated bacterial strains, *Vibrio mimicus* observed to be the effective alkaline protease producer. Identification was done based upon biochemical test and 16s rRNA gene sequencing. This strain could produce high alkaline protease in medium containing 2% NaCl in 2days. The optimum pH and temperature for maximal protease activity was 9.0 and 40°C, respectively. The optimum protease production was achieved with 0.5% lactose and 0.5% yeast extract added medium. Among the inorganic nitrogen sources used, the protease production was supported by the addition of potassium nitrate. In experimentation with metal ions, the maximum protease production was observed (863.44 ± 1.63 U/ml) in the media supplemented with magnesium chloride. The maximum amount of protease production was obtained in Triton X 100 (309.275 ± 1.63 ml) added medium when compared to the other tested surfactants.

Keywords: Protease, *Vibrio mimicus*., Optimization. 16s rRNA gene sequence

1. Introduction

Microflora of the digestive tract of fish may produce antibacterial materials preventing pathogenic bacteria from getting into an organism. Further it has also been reported that aquatic microbes synthesize exoenzymes identified as inducible catabolic enzymes (Bhaskar et al., 2006). In recent years, proteases from the gut of fishes received much attention (Chi *et al.*, 2007). Microbial proteases are an important group of enzymes that can have application in various industries such as leather processing, food processing, pharmaceutical and bioremediation process. Moreover, protease production is influenced by some physical factors such as aeration, inoculum size, pH, temperature and incubation time and biological factors such as the genetic nature of the organism, which are influencing the metabolic and biochemical behavior of the microbial strain. In general, no defined medium has been established for the best production of any metabolite, because the genetic diversity exist in microbial sources causes each organisms or strain to have its own special conditions for the maximum production of protease. Therefore, it is worth to have a detailed investigation on newly isolated microbial strain for production pattern under different environmental conditions and in an optimized pattern to achieve maximum production (Elliah *et al.*, 2002). Hence the present work was undertaken to investigate the protease production by the fish gut bacterial isolate *Bacillus licheniformis*.

2. Materials and Methods

2.1. Isolation and screening of proteolytic bacterial strain

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Protease producing bacterial strain was isolated from the gut of *Penaeus monodon*. The isolated organisms were plated on skim milk agar plates and the plates were incubated at 35°C for 24h. A clear zone formation of skim milk hydrolysis gave an indication of protease producing organisms. Depending upon the production of haloalkalophilic protease by qualitative and quantitative assay, the candidate species was selected for further experimental studies. The protease production in the liquid medium was assessed first by enriching the bacteria in enrichment medium containing beef extract (0.3 %), peptone (0.5 %), NaCl (1 %) and glucose (0.5 %) at pH 9 for 24 h. The culture was then incubated for 2 days by shaking at 35°C. The cells were then harvested by centrifugation at 10000 rpm for 15 min and the supernatant was used for further protease assay.

2.2. Protease assay

The assay system consists of following ingredients such as 1.25 ml Tris buffer (pH 7.2), 0.5 ml of 1% aqueous casein solution and 0.25 ml culture supernatant. Approximate controls were also made with out enzyme source. The mixture was incubated for 30 min at 35°C. Then 3 ml of 5% TCA was added to this mixture and placed at 4°C for 10 min to form precipitate. Then it was centrifuged at 5000 rpm for 15 min. From this, 0.5 ml of supernatant was taken, to this 2.5 ml of 0.5M sodium carbonate was added, mixed well and incubated for 20 min. Then it was added with 0.5 ml of folin phenol reagent and the absorbance was read at 660 nm using UV-Vis Spectrophotometer (TECOMP 8500). The amount of protease produced was estimated and expressed in microgram of tyrosine released under standard assay conditions.

2.3. Strain identification

Identification of selected isolate was studied based on different morphological, physiological and biochemical characteristics. This data was compared with standard description given in Bergy's Manual of Systematic Bacteriology (Holt *et al.*, 1994)

2.4. Optimization of media components

To optimize the culture conditions for maximum production of protease different parameters such as incubation periods (12, 24, 48, 72, 96 and 120h), medium pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10), temperature (10°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C and 80°C), carbon sources (galactose, sucrose, arabinose, fructose, maltose, raffinose, starch, glucose and dextrin) organic nitrogen sources (casein, peptone, beef extract, urea, yeast extract, soy meal and skim milk powder), inorganic nitrogen sources (ammonium sulphate, ammonium chloride, sodium nitrate, ammonium nitrate and potassium nitrate), sodium chloride (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5% and 4%), surfactants (Tween 20, Tween 40, Tween 60, Tween 80, Triton X 100, (PEG) Poly Ethylene Glycol and (SDS) Sodium Dodecyl Sulphate), metal ions (calcium chloride, ferric chloride, mangnous chloride, EDTA, copper sulphate, zinc chloride, zinc sulphate and mercuric chloride) were used. The effect of these parameters on protease production was recorded.

3. RESULTS

3.1. Isolation, screening and identification of haloalkalophilic of protease producing bacterium

A total of ten bacterial strains were isolated from the gut of *Penaeus monodon* and we found that the strain *Vibrio mimicus* can be used to produce the maximum haloalkalophilic protease and it was selected for the subsequent studies. Based on the biochemical tests the suspected colony was identified as *Vibrio mimicus* by the following standard keys of Bergey's Manual of Determinative Bacteriology.

3.2. Effect on incubation time on protease production

When the isolate *Vibrio mimicus* was grown in liquid culture medium to determine optimum incubation time was carried out at 1 - 4 days and maximum protease production was recorded with 2 days incubation period. This report was supported by earlier study of Shanmuga Priya *et al.* (2008). Who reported that the maximum alkaline protease production by marine bacterium *Roseobacter* sp after 48h of incubation (Fig 1).

3.3. Effects of temperature and pH on protease production

The production profile of protease against temperature shows that the highest yield (252.60 ± 2.04 U/ml) at 40°C . This report was more or less similar to the earlier report of Wang *et al.* (2006). They reported that the protease production was high in 45°C by marine yeast (Fig. 2). This report is in accordance with the earlier report of Shanmuga priya *et al.* (2008). They have reported that the production of alkaline protease by marine *Roseobacter* sp was grown at 37°C . Effect of pH on protease production was determined by incubating the culture conditions between the pH 3.0 to 10 (Fig. 3) and it was optimum at (352.13 ± 1.47 U/ml) pH 9.0. This report was identical to the previous report of Agarwal *et al.* (2005). They suggested that the *Beauveria feline* was optimal active at pH 7.0 for the protease production.

3.4. Effect of nitrogen and carbon sources

Among the organic nitrogen sources used, yeast extract (375.8 U/ml) had the significant effect on the extracellular protease production and beef extract (161.47 U/ml) exhibited poor effect on the protease production (Fig. 4). Similar observations were noticed in case of alkaline protease production by *Bacillus* sp (Gouda *et al.*, 2006). Many researchers have been shown that different carbon sources have different influences on the protease production of extracellular enzymes and the growth of different strains (Chi and Zhao, 2003). Among the various carbon sources tested, lactose was observed to be the effective source for the protease production of this strain (408.40 ± 1.63 U/ml) *Vibrio mimicus* (Fig. 5). Increased yield of alkaline protease production were reported by several other researchers who used different sugars such as lactose, maltose, sucrose and fructose (Malathis and Chakraborty, 1991).

3.5. Effect of salt concentration, surfactants and metal ions

The effect of NaCl on protease production by *Vibrio mimicus* inferred that, it was high (410.92 ± 1.63 U/ml) in 2% concentration (Fig. 6). From this results it can be conclude that the protease production by marine bacterium *Roseobacter* sp. absolutely require 3% NaCl (Shanmuga Priya *et al.*, 2008). Result on surfactants induced protease production after 48 h of incubation indicated that, triton X 100 (309.275 ± 1.63 ml) added medium produced maximum protease. This report is contrary with the report of Joo and Chang (2005) that the protease from *Bacillus clausii* and *Bacillus* sp. retain their activity with different surfactants such as Triton X 100, Tween 20 and SDS. Among the tested metal ions, the maximum amount of enzyme production was recorded in magnesium chloride (863.44 ± 1.63 U/ml) added medium after 48 h of fermentation (Fig.9). Sumantha *et al.* (2005) reported that, some bacteria and fungi showed maximum enzyme production with metal ions such as Ca^{2+} , Mg^{2+} supplemented medium.

4. References

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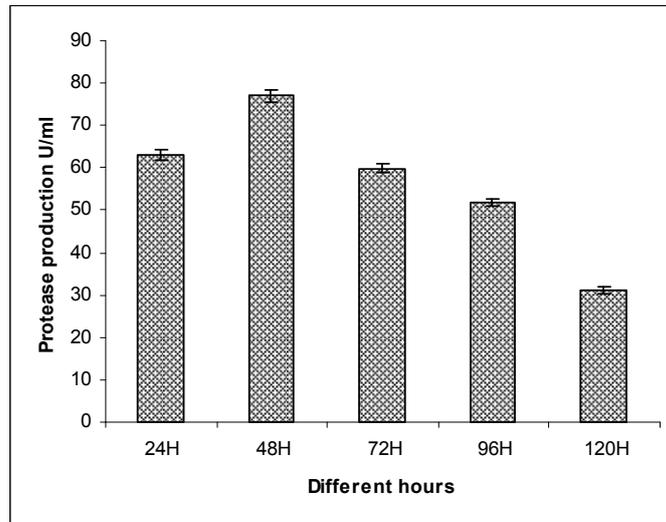


Fig. 1 Production profile of protease produced by *Vibrio mimicus* in various incubation time at 35°C for 48 h incubation

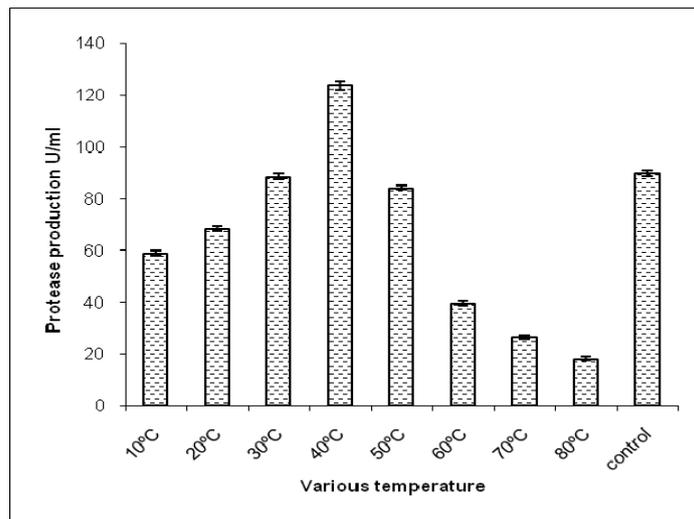


Fig. 2 The effect of various temperatures on protease production. The cells were incubated in various temperatures at 48 of incubation by *Vibrio mimicus*

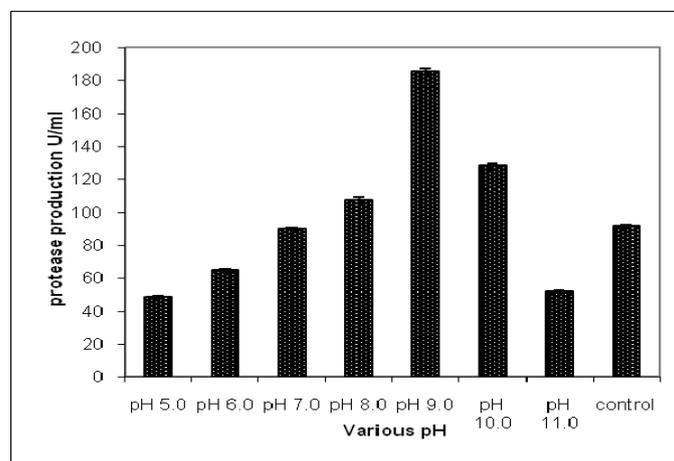


Fig. 3 Effect of various pH on protease production. The cells were cultivated in medium with various pH by *Vibrio mimicus* at 35°C for 48 h incubation

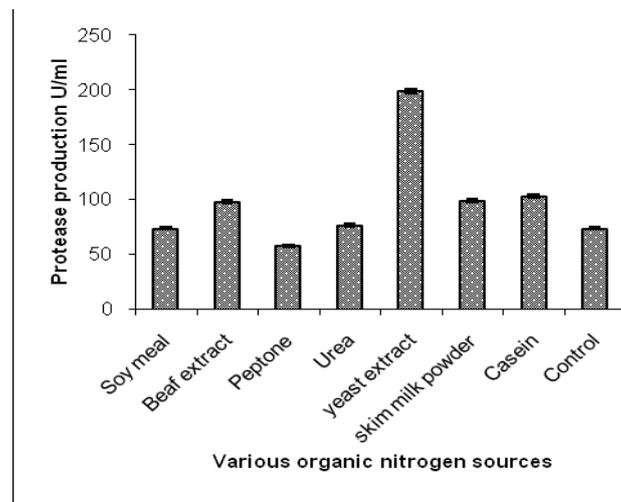


Fig. 4 Effect of various organic nitrogen sources on protease production by *Vibrio mimicus* in basal medium supplemented with 0.5% nitrogen sources at 35°C for 48 h incubation

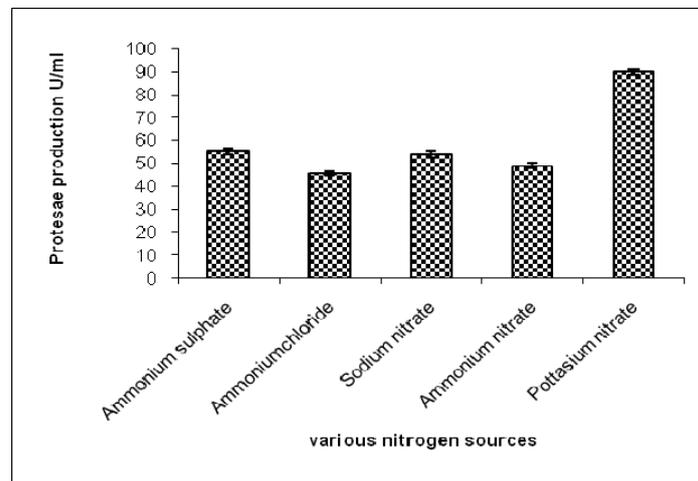


Fig. 5 Effect of various organic nitrogen sources on protease production by *Vibrio mimicus* in basal medium supplemented with 0.5% nitrogen sources at 35°C for 48 h incubation

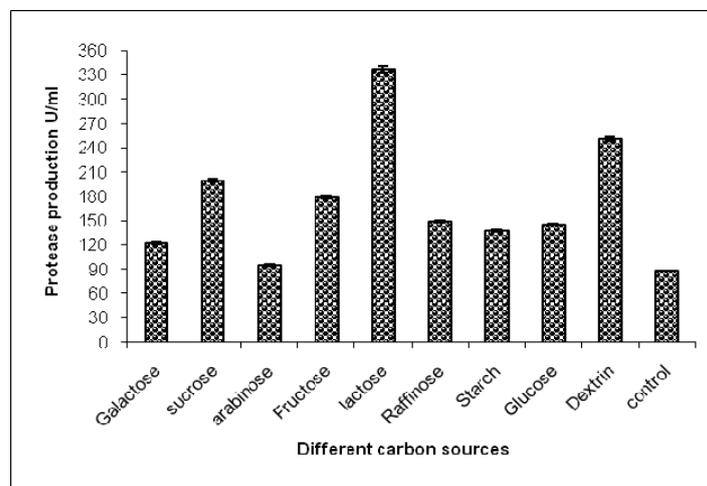


Fig. 6 Effect of various carbon sources on protease production by *Vibrio mimicus* in basal medium supplemented with 0.5% carbon sources at 35°C for 48 h incubation

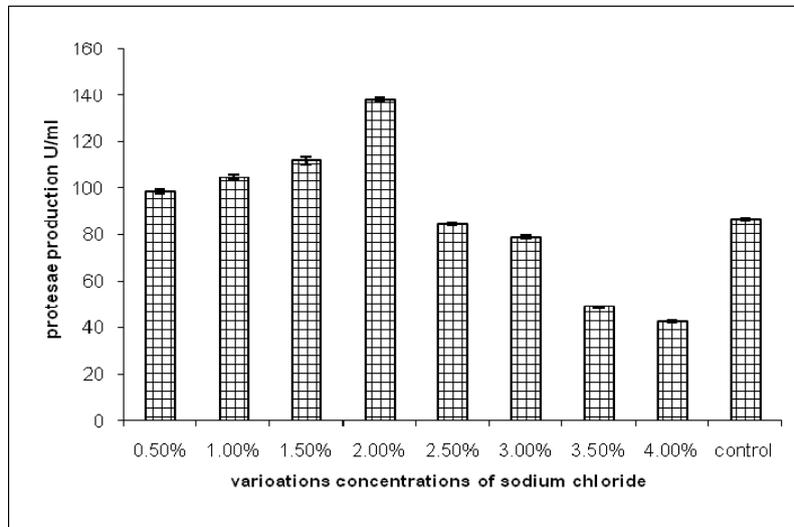


Fig. 7 Production profile of protease in medium supplemented with various concentrations of sodium chloride *Vibrio mimicus* at 35°C for 48 h incubation

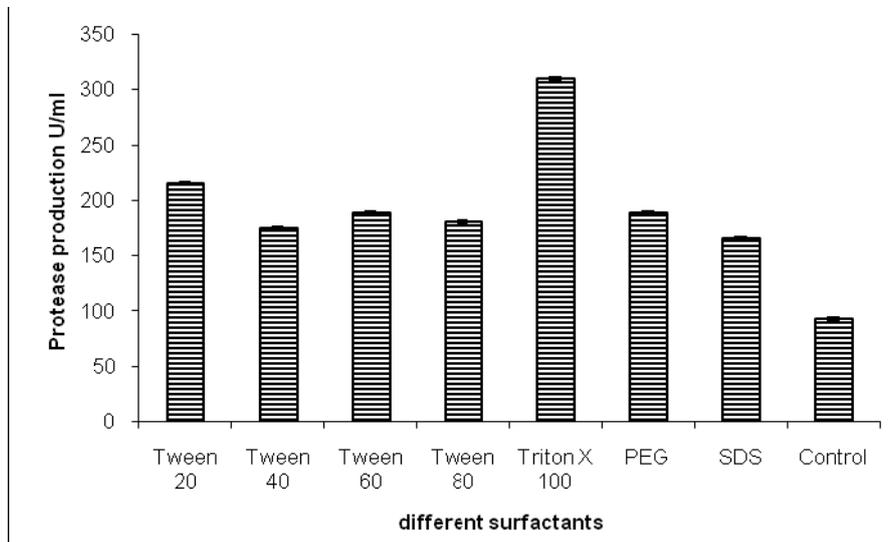


Fig. 8 Production profile of protease in medium supplemented with various kinds of surfactants by *Vibrio mimicus* at 35°C for 48 h incubation

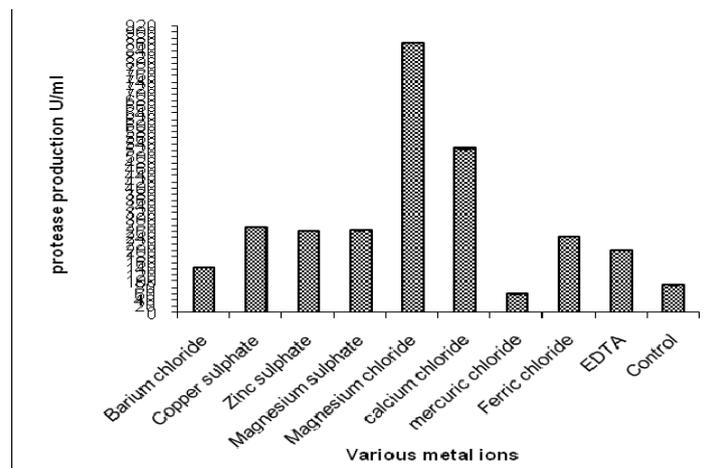


Fig. 9 Production profile of protease in medium supplemented with various kinds of metal ions by *Vibrio mimicus* at 35°C for 48 h incubation