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Scaleup and media optimization of protease by *Vibrio alginolyticus*

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Keywords

Vibrio alginolyticus
Protease activity
Carbon source
Organic nitrogen source
Scaleup
Molecular weight

Abstract

The growth and protease production by *Vibrio alginolyticus* was examined in the present investigation. The maximum protease activity was 2037U/ml using 1% (w/v) of potato peel extract as the carbon source. Potato peel is an inexpensive and readily available domestic waste and thus it can be used as the cost effective crude material for the production of an extracellular protease. The organic nitrogen source, fish scrap powder proved to be an effective organic nitrogen source, where the protease activity was 1374 U/ml. A maximum protease activity of 620(U/ml) in aerobic and 368 (u/ml) in anaerobic condition were produced in 24h in a 3L bioreactor respectively. The enzyme acted optimally at pH 8.8 and temperature 36˚C. 2% NaCl concentration was suitable for efficient enzyme production. The activity of the enzyme was inhibited by all the inhibitors tested.. In the growth curve studies was found to be fast growing. Among the metal ions tested calcium chloride and magnesium chloride enhanced the protease by 7%. The molecular weight of the enzyme was determined as 38KDa. The enzyme retained more than 89% of its activity after 60 min of incubation at 40˚C in the presence of various detergents.

1. Introduction

With increasing emphasis on environmental protection, the use of enzymes particularly from extremophiles has gained considerable attention during the last several years. Extremozymes are now replacing chemical catalysts in many industries including manufacturing of chemicals, textiles, pharmaceuticals, etc. Alkaliphiles are reported to be a rich source of alkaline active enzymes, example, amylase, protease, cellulose and xylanase and other enzymes that have numerous application in many industrial processes (Horikoshi, 1999, oskouie *et al*., 2008).

Vibrio alginolyticus is a gram negative rod-shaped marine bacterium. *Vibrio* species is commonly isolated from clinical samples on TCBS agar plates on which it grows as bluish-green colony (Chowdary *et al.,* 1990). *Vibrio* species produce many enzymes like amylase, gelatinase, chitinase and DNase. Some Vibrio species are halophilic (tolerance upto 10% Nacl) and sodium ions stimulate their growth. Vibrios grow well in neutral to alkaline pH 9.0 and are acid sensitive. The optimum pH range is 8-8.8 and the optimum growth temperature is 20-37̊ C. A chromogenic agar medium has recently been developed for effective detection of *Vibrio* species from seafood samples (Hara Kudo *et al.,* 2001). *Vibrio* species produces alkaline proteases.

Proteases, also known as peptidyl-peptide hydrolysis, (EC 3.4.21-24 and 99) constitute 60 to 65 % of the global enzyme market (Banerjee *et al*., 1999; Genckel and Tari, 2006; Laxman *et al*, 2005) proteases are the important tool for studying the structures of proteins and peptides. Besides that they are also used in medical diagnosis and decomposition of gelatin on x-ray films as well as in textiles (Joo *et al*., 2002; Patel *et al*., 2005; Tari *et al*., 2006). In general, proteases constitute a very large and complex group enzyme, which differs in the properties such as substrate specificity, active site and catalytic mechanism, pH and temperature activity and stability profiles. The inability of the plant and animal proteases to meet the demands led to and increased interest in microbial proteases.

Alkaline proteases are an important group of industrial enzymes and are produced by a wide range of micro organisms including fungi, animal and bacteria. Bacteria which produce proteases include *Vibrio alginolyticus*, *Alkaligenes faecalis*, *Pseudomonas flourescens* and *Aeromonas hydrophilia*. Proteases execute a large variety of function and have numerous applications in detergent, food, pharmaceutical and leather industries. The largest application of the proteases is in the laundry detergents, when removing protein-based strains from clothing during washing. The enzymes to be used as detergent additives should be stable and active in the presence of typical detergent ingredients.

For the bulk production of commodity product like enzymes, the cost of production media can substantially affect the overall process economics. Approximately 40% of the production cost of industrially important enzymes is estimated to derive from the cost of growth medium (Joo *et al*., 2002).Thus the use of cost effective growth medium for the production of alkaline proteases from alkalophilic *Vibrio alginolyticus* is especially important, because the enzymes account for approximately 25% of the world wide enzyme consumption(Gessesse,1997).

The preliminary experiments revealed that *V. alginolyticus,* is highly efficient in producing alkaline protease and hence in the present study, the investigations are done for the production of effective alkaline protease in the low cost medium by *V. alginolyticus.*

2. Materials and Methods

Chemicals

Peptone, beef extract, NaCl, casein, TCA, Folin's ciocalteau phenol reagent, sodium carbonate, ammonium phosphate, etc used were of analytical grade and were produced from local suppliers.

Organism and Growth maintenance

Vibrio alginolyticus, a potent protease produce was obtained from the microbiology laboratory of PRIST University and selectively screened on skim milk agar plate. The organism was cultivated at 37±1°c in the incubator for 24 hrs and subsequently maintained at 4°c in a biological oxygen demand incubator by routine transfers after every 15 days on nutrient agar slants at pH 9.

Protease production medium

The basal medium used for protease production contained (g/L) beef extract (0.5), peptone (0.3), NaCl (0.5).The production medium (50ml) was inoculated with 1% of inoculum. The culture was incubated at 37±1°c at 150rpm with pH 9 in an incubator shaker. After a time period of 24 hr, the culture was centrifuged at 3000xg for 20 minutes. The supernatant was used as the protease source.

Preparation of tyrosine standard

0.05, 0.1, 0.15 and 0.2ml of 1.1mM L-tyrosine was taken in 4 separate test tubes.5ml of 500mM Na_2CO_3 and 1ml of diluted Folin's-ciocalteaus phenol reagent was added in the test tubes. The total volume was made up to 8ml using distilled water. Finally, the absorbance was measured calorimetrically at 620nm by keeping L-tyrosine as standard. (Praveen Kumar *et al*., 2008).

Protease assay

The proteolytic activity was measured by the photometric method of Rahman et al., 2007.In this method, the proteolytic activity of enzyme was determined using casein as a substrate. Casein was dissolved in 0.1M Tris-HCl buffer (pH 9) at a concentration of 1%. The assay mixture consisted of 1ml of substrate (casein) and 5ml of enzyme solution suitably diluted with 0.1M Tris-HCl buffer (pH 9). The reaction mixture was incubated at 37ºC for 10 min and the reaction was terminated by the addition of 500µl of 10% trichloro acetic acid (TCA) and then centrifuged at 5000Xg for 15 min to remove resulting precipitate. Protease activity was determined as the tyrosine released from the supernatants. One unit of enzyme activity was defined as the amount of the enzyme resulting in the release of 1µg of tyrosine per min at 37ºC under the reaction conditions (Joo *et al*., 2002). Growth content was evaluated by calorimeter as optical density at 620nm.

Determination of protease activity

Proteolytic activity is represented in terms of U/ml enzyme and is derived by:

$$\frac{\text{µmole tyrosine equivalents released X total volume (ml) of assay}}{\text{Volume of enzyme (ml) X time of assay (min) X volume used in calorimeter (ml)}}$$

One unit (Anson *et al*., 1938) of enzyme will hydrolyze casein to produce color equivalent (Folin *et al*., 1929) to 1.0 µmole (181 µg) of tyrosine per minute at pH 8.0 at 37°C (color by Folin and ciocalteau's reagent). The µmoles of tyrosine equivalents librated were calibrated by using the standard curve. After evaluation, the protease activity was determined by the above formula mentioned.

Effect of carbon source

The effect of carbon source on the production of protease by *Vibrio alginolyticus* was determined using different carbon sources that comprises of potato peel extract, beet root peel extract, carrot peel extract and sweet potato peel extract.

Effect of organic nitrogen source

The effect of various organic nitrogen sources was tested on the production of protease by *Vibrio alginolyticus* the organic nitrogen sources such as fish scarp powder, crab shell powder, prawn shell powder and skim milk powder.

The results obtained using the above carbon-nitrogen sources were compared to that obtained from protease produced using nutrient broth. The above mentioned carbon and nitrogen sources were replaced in the basal medium (beef extract, peptone, NaCl) at a concentration of 1%

(w/v) respectively. Protease yield was determined after 24h of incubation at 37°C under shaking condition of 150 rpm.

ENZYME CHARACTERIZATION

Effect of temperature on enzyme stability

The effect of temperature on the stability and activity of the protease was determined by incubating the enzyme at different temperature ranging from 35-40°C.

Effect of pH on enzyme stability

The pH stability and activity of enzyme was tested at different pH ranging from 8-9 using phosphate and Tris-HCl buffer.

NaCl tolerance

The effect of salt on the growth of the organism and enzyme activity was studied by growing the organism in the production media supplemented with various concentrations of NaCl (1-7%) at 37°C in an incubator shaker at 120 rpm. Protease production was monitored at regular intervals.

Effect of various inhibitors on protease activity

The effect of various inhibitors on the enzyme was studied by incubating the enzyme with different inhibitor compounds for 30 min at room temperature and the relative activity was determined by standard assay protocols. All the inhibitors were used in 1mM final concentration.

Effect of metal ions on protease activity

The effect of metal ions of 1mM concentration on protease activity was determined by incubating the enzyme with different metal salts at room temperature for 1h and thereafter, the relative activities were determined under standard assay conditions.

SCALE UP OF PROTEASE

Aerobic condition

Cultivation of *Vibrio alginolyticus* for protease production was also carried out in a 3L fermentor (Batch fermentor) with a working volume of 2.5L. The medium containing potato peel extract as the carbon source and fish scrap powder as the nitrogen source along with 2% NaCl and $CaCl_2$ as the inducer was sterilized insitu and was inoculated with appropriate size of seed inoculums (25%). The fermentation was carried out at 36±°C for 24h with controlled pH at 8.8. The impeller speed was initially adjusted to 150 rpm and compressed sterile air was sparged into the medium at constant rate of 0.5vvm. Samples were withdrawn periodically at an interval of 3h and analyzed for protease production. The fermentation parameters such as temperature, pH and air flow rate were continuously monitored.

Anaerobic condition

Cultivation of *Vibrio alginolyticus* for protease production was also carried out in a 3L fermentor (Batch fermentor) with a working volume of 2.5L. The medium containing potato peel extract as the carbon source and fish scrap powder as the nitrogen source along with 2% NaCl and $CaCl_2$ as the inducer was sterilized insitu and was inoculated with appropriate size of seed inoculums (25%). The fermentation was carried out at 36±°C for 24h with controlled pH at 8.8 without aeration. Samples were withdrawn periodically at an interval of 3h and analyzed for protease production. The fermentation parameters such as temperature, pH and air flow rate were continuously monitored.

Partial purification of protease

The obtained protease was partially purified by "Ammonium sulfate fractionation" method. In this method, the volume of the protein solution was measured and taken in beaker. This is placed in the ice bath. The required amount of ammonium sulphate is calculated, taking 0.6gm of salt per ml of the protein solution. After complete dissolution of salt into the enzyme solution, it is centrifuged at 5000 rpm and the pellet is collected which the partially purified enzyme.

Molecular weight determination

The partially purified enzyme mixture was subjected to electrophoresis on 10% acryl amide gel and the molecular weight was determined by comparing the relative mobility of the protein band with that of molecular weight marker

Compatibility with commercial detergents

Detergent solutions at a concentration of 10μg/ml were prepared in double distilled water. The solutions were boiled for 10 min to destroy any protease already present and then cooled. 0.5ml of partially purified enzyme was added to each detergent solution and the mixture was incubated at 37°C for different time intervals. The activity was then assayed by modified Lowry's method.

3. Results

Protease production in the basal medium

The selected organism *Vibrio alginolyticus* exhibited a large zone of hydrolysis on milk agar plate and was taken up for optimization of maximum production, scale up and protein characterization. The isolate was a gram positive, motile, rod shaped bacterium and strictly aerobic.

This isolate produced 420 U/ml of protease in the initial production medium.

It is well documented in the literature that nitrogen is metabolized to produce primary 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-nitrogen sources available in the medium (Chu et al., 1992; Moon et al., 1991; Patel et al., 2005).

Effect of carbon and nitrogen sources

Vibrio alginolyticus produced maximum protease in potato peel extract (2031U/ml) followed by beetroot extract (1905U/ml). However, the other two carbon sources, carrot peel extract (1685 U/ml) and sweet potato peel extract (1346 U/ml) did not show any remarkable increase in the enzyme production (Figure 1).

Fig. 1. Ammonium Sulphate Precipitation

The organism was grown in the presence of different organic nitrogen sources, replacing the total nitrogen from the production medium with equivalent amount of nitrogen, in the presence of 0.5% potato peel extract as the carbon source. All the organic nitrogen sources used in the study supported growth and production. Maximum production of protease (1374 U/ml) was observed with 1% fish scrap powder (Figure 2). This was followed by prawn shell powder (1363 U/ml), crab shell powder (1100 U/ml) and skim milk powder (1093U/ml).

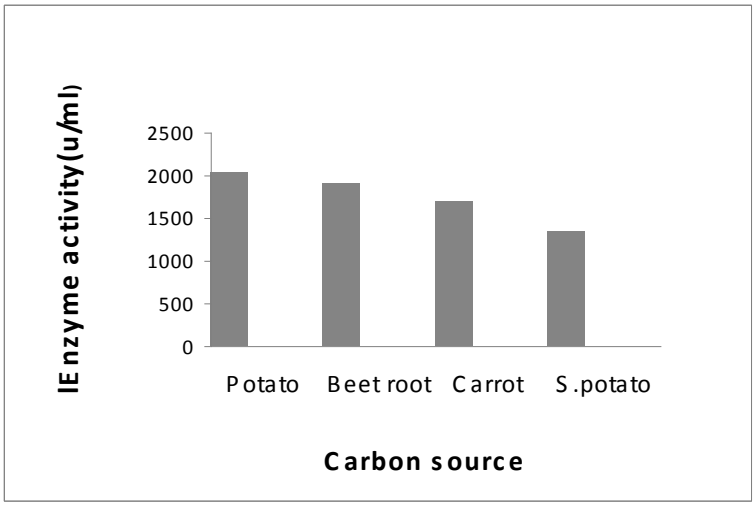

Fig. 2. Effect of carbon source on protease

Enzyme characterization

Effect of temperature

The data represented in the Figure 3 exhibit the effect of different temperature on the protease activity. Temperature affects not only the growth of the organism, but also the production of enzyme and the enzyme activity. *Vibrio alginolyticus* exhibited its maximum protease activity between 40˚C and 45˚C. The enzyme lost its activity above 50˚C.

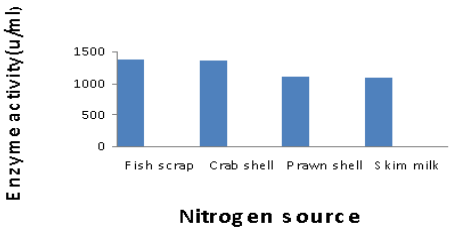

Fig. 3. Effect of nitrogen source on protease

Effect of pH

Data presented in Figure 4 showed the effect of different pH on the protease activity. Maximum protease activity was exhibited at pH range between 8 and 9.

NaCl tolerance

The alkaline protease production by *Vibrio alginolyticus* was studied within the range of 1-7% Nacl concentration, but maximum production was observed at 2% concentration (Figure 5).

Effect of inhibitors

The activity of alkaline protease was completely inhibited in the presence of 1mM Phenyl Methyl Sulfonyl Fluoride (PMSF). Other inhibitors like bestatin, chymostatin, β-mercapto

ethanol and Ethylene Di Amine Tetra Acetate (EDTA) also inhibited the enzyme activity to some extent (Figure 7).

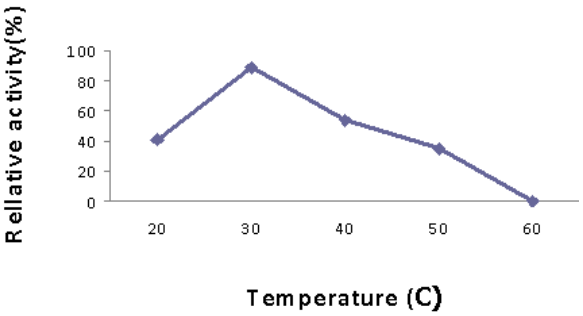

Fig 4.Effect of temperature on protease

Effect of metal ions

Among the metal ions tested, calcium chloride ($CaCl_2$) and magnesium chloride ($Mgcl_2$) marginally stimulated the protease activity up to 5% at 1mM concentration (Figure 6). These cations probably protect the enzyme against thermal denaturation and therefore maintain the active conformation of the enzyme even at high temperature. Others like zinc chloride ($ZnCl_2$), copper sulphate ($CuSO_4$) and urea also inhibited the protease activity.

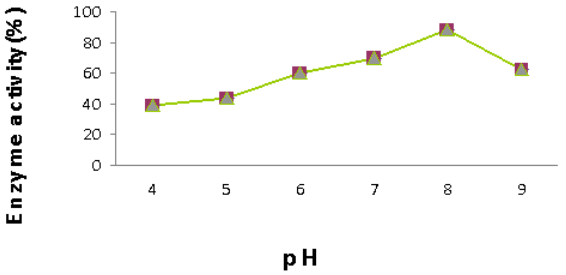

Fig. 5.Effect of pH on protease

Growth curve studies

During this study, the organism was found to be fast growing. The maximum proteolytic activity (89%) was observed after 24h of growth where it reached stationary phase. The alkaline protease activity was stable even after 48 hours of growth (Figure 8) and drastically reduced 72hour.

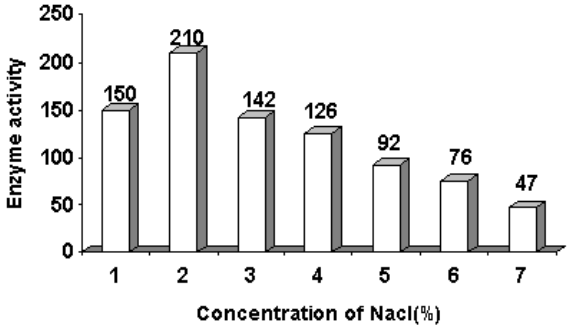

Fig 6. Effect of NaCl tolerance

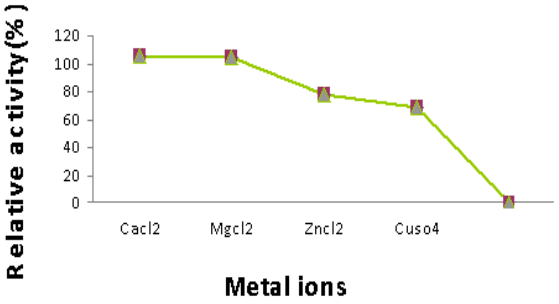

Fig 7.Effect of metal ions on protease

Scaleup of protease

Aerobic condition

Scale up studies was carried in a 3L bioreactor (batch reactor) with a working volume of 2.5L. Protease production started early within 6h, however, there was no significant increase in the production up to 10h.This is because, at this time, the organism was in the exponential phase. The exponential phase was followed by the stationary phase where in the maximum protease activity was 2308 U/ml in 24h (Figure 9). Thereafter, there was a decline in protease production in the bioreactor.

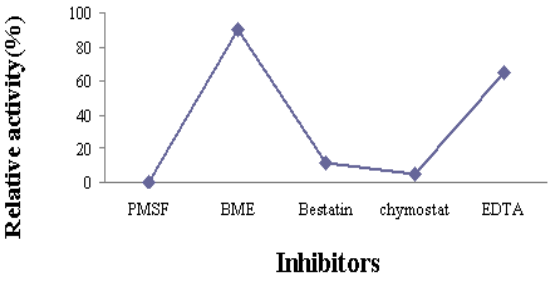

Fig 8.Effect of inhibitors on protease

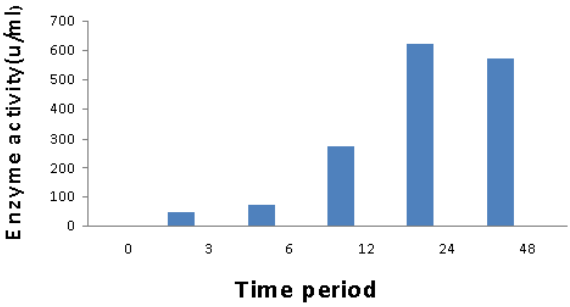

Fig 9. Scaleup protease in aerobic condition

Anaerobic condition

The fermentation was carried out at 37˚c for 24 hours with controlled pH 8.8 and 130 rpm without aeration. Protease production started early within 6h, however, there was no significant increase in the production up to 10h.This is because, at this time, the organism was in the exponential phase. The exponential phase was followed by the stationary phase where in the

maximum protease activity was 2308 U/ml in 24h (Figure 10). Thereafter, there was a decline in protease production in the bioreactor.

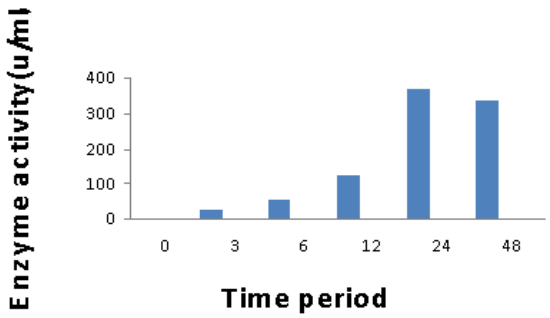

Fig 10.Scaleup of protease in anaerobic condition

Partial purification

The produced enzyme was partially purified by ammonium sulphate precipitation method (Figure 11). The crude enzyme was found to be 60% pure. Further purification can be done by various chromatographic methods to obtain 100% pure enzyme.

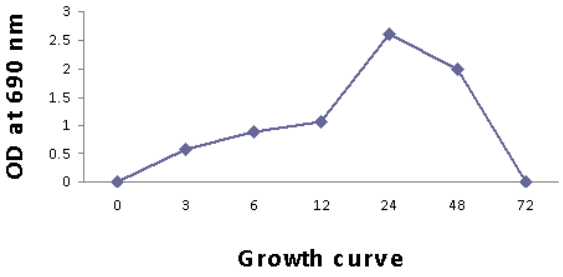

Fig. 11. Growth curve on Vibrio alginolyticus

Molecular weight determination

Electrophoresis on SDS-PAGE revealed a single band of protein corresponding to a molecular weight of 38KDa (Figure 12).

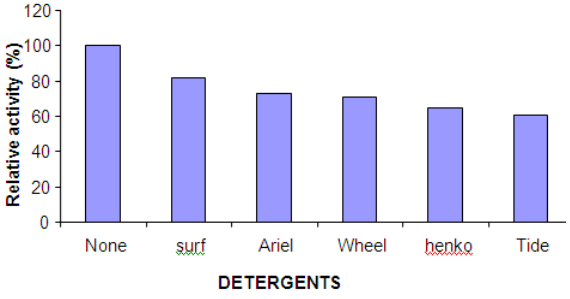

Fig.12.Compatibility with detergents

Compatibility with various commercial detergents

Enzyme activity and stability in presence of some available commercial detergents was studied with a view to exploit the enzyme in the detergent industries. The enzyme retained 80-90% of its original activity in various detergents (Figure 13).

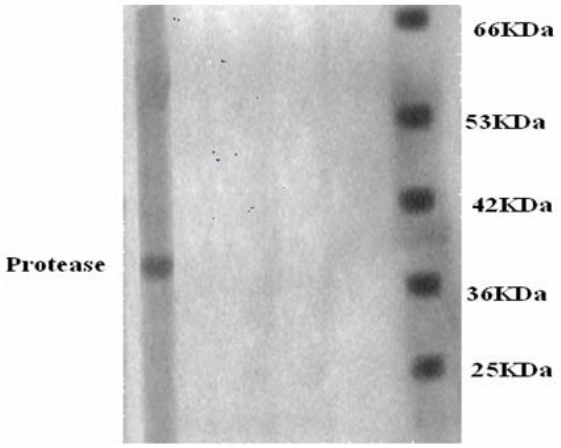

Fig. 13. Determination of molecular weight for protease

4. Discussion

The selected organism *Vibrio alginolyticus* exhibited a large zone of hydrolysis on milk agar plate and was taken up for optimization of maximum production, scale up and protein characterization. The isolate was gram positive, motile, rod shaped bacterium. The isolate produced 420 U/ml of protease in the initial production medium.

It is well documented in the literature that nitrogen is metabolized to produce primary 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-nitrogen sources available in the medium (Chu *et al.*, 1992; Moon *et al.*, 1991; Patel *et al.*, 2005). In our present study, *Vibrio alginolyticus* produced maximum protease in potato peel extract (2031 U/ml) followed by the other extracts. In the similar study conducted by Prakasham *et al.*, 2005, they used green gram husk as the carbon source for the efficient production of protease by *Vibrio alginolyticus*, Sourabh *et al.,* 2007, used soybean as the substrate for the production of alkaline protease by *Vibrio alginolyticus*. A maximum of 3208 U/ml of enzyme was produced in their experiment.

Among the 4 different nitrogen sources used here, fish scrap powder, an organic nitrogen source, proved to be the best nitrogen source with the enzyme production at the maximum of 1364 U/ml. Nilegoankar *et al.*, 2007 reported that the protease activity was highest with soy bean meal (124.4 U/ml) as the nitrogen source. Deshpande *et al.* (2004), also reported that soybean meal (organic nitrogen source) was used as an inducer for protease production from *conidiobolus coronatus*. Our results are in accordance to the findings of Ferrero *et al.*, 1996, Johnvesly and Naik (2000); Kole *et al.*, 1988, who observed that inorganic nitrogen sources

are less favourable for the growth and enzyme production.

The temperature was an important environmental factor that affects the growth of the microorganisms as well as production of enzymes. *Vibrio alginolyticus* exhibited its maximum protease activity between 35-40°C. This was correlated with the results obtained by other workers, who found that the maximum protease production occurred at 45°C for *Bacillus thermoruber,* a thermophillic *Bacillus sp.* and *Bacillus horikoshi* respectively (Fujiwara *et al.*, 1993).

The organism produced maximum protease at a pH ranged 8.5-9. This is in complete contrast with the findings of Remeikaite, 1979; Do Nascimento and Martin, 2004 and Wang *et al.* (2006). According to their findings the optimum pH for the maximum protease production was 7.5 for *B. subtilis*, 7.2 for *B. subtilis* k11 and *B. licheniformis* and 7 for *Bacillus* Sp. TKU004. The alkaline protease production by *Vibrio alginolyticus* was studied within the range of 1-7%. NaCl concentration and the maximum production were observed at 2% concentration. This was in accordance with the findings of Kiranmayee Rao and Lakshmi Narasu (2007), in which they said that 2% Nacl is the best concentration for protease production. The activity of alkaline protease was completely inhibited in the presence of 1mM phenyl methyl sulfonyl fluoride (PMSF). Other inhibitors like bestatin, chymostatin, β-mercapto ethanol and ethylene di amine tetra acetate (EDTA) also inhibited the enzyme activity to some extent. This observation is in agreement with the results of Huang *et al.*, 2003 and Tang *et al.*, 2004, who said that PMSF is known to inhibit the serine residue at the active site causing complete loss of the enzyme.

Among the metal ions tested, calcium chloride ($CaCl_2$) and magnesium chloride ($Mgcl_2$) marginally stimulated the protease activity up to 5% at 1mM concentration. These cations probably protect the enzyme against thermal denaturation and therefore maintain the active conformation of the enzyme even at high temperature. Others like zinc chloride ($ZnCl_2$), copper sulphate ($CuSO_4$) and urea also inhibited the protease activity. Tsuchiya *et al.*, 1987, reported that protease isolated from Colosporium Sp. KM388 was inhibited by Hg^{++}. Kaur *et al.*, 1998, found that Mn^{++}, Cu^{++} and Ca^{++} inhibited the enzyme activity of alkaline protease secreted by *B. polymyxa*. Similarly Nehra *et al.*, 2004, reported that Mg^{++} was found to be an activator of alkaline protease produced by *Aspergillus* Sp. In a study conducted by Kiranmayee Rao and Lakshmi Narasu (2007), the maximum proteolytic activity (215 U/ml) by *Bacillus firmus* MTCC7728 was observed after 48h of growth. In the present investigation, *Vibrio alginolyticus* showed maximum proteolytic activity (256 U/ml) after 24h of growth where it reached stationary phase.

Scale up studies was carried in a 3L bioreactor (batch reactor) with a working volume of 2.5L. Protease production started early within 6h, however, there was no significant increase in the production up to 10h.This is because, at this time, the organism was in the exponential phase. The exponential phase was followed by the stationary phase where in the maximum protease activity was 620 U/ml in 24h in aerobic condition and 368 U/ml in anaerobic condition respectively. Thereafter, there was a decline in the protease production in the bioreactor. This was nearly similar to the data obtained by Sourabh *et al.*, 2007, who used 10L bioreactor with 7.5L working volume for the production of alkaline protease from *Bacillus sp.* The produced enzyme was partially purified by ammonium sulphate precipitation method. The crude enzyme was found to be 60% pure. Further purification can be done by various chromatographic methods to obtain 100% pure enzyme. The same method was used for the purification of thermostable protease produced by *B. brevis geltinoamylolyticus* (Ammar *et al.*, 2003). Moreover, Secades (2001), purified the extracellular protease from *Flavobacterium psychrophilum* by using ammonium sulphate precipitation, ion exchange chromatography, hydrophilic chromatography and size exclusion chromatography. Electrophoresis on SDS-PAGE revealed a single band of protein corresponding to a molecular weight of 38KDa.

The molecular weight in the range of 32-33 KDa has also been reported by Voordouw *et al.*, 1974, for the enzyme obtained from *Malbranchea inlchella.* This result also in harmony with those of Kalpana Devi *et al.*, 2008, who purified and characterized alkaline protease from *Aspergillus niger*, with a molecular weight of 38 KDa. Considering these facts, it is concluded that, *Vibrio alginolyticus*, proved to be an efficient strain in producing alkaline protease. Potato peel extract and fish scrap powder was found to be the best carbon and nitrogen source respectively. 2% NaCl concentration favoured the efficient enzyme production. The optimum pH and temperature were 8.8 and 36°C respectively. The enzyme retained its efficiency up to 70% when tested along with commercial detergents. Hence, from the obtained results, the optimized environmental conditions could be used in further studies.

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