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Media Optimization and Scaleup of Protease by *Bacillus coagulans* MTCC 492

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Keywords

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Abstract

The growth and protease production by *Bacillus coagulans* MTCC 492 was examined in the present investigation. The maximum protease activity was 127 U/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 227 U/ml. A maximum protease activity of 2308 U/ml was produced in 24h in a 3L bioreactor. The molecular weight of the enzyme determined by SDS-PAGE was 36 KDa. The enzyme acted optimally at pH 9 and temperature 45°C. 2% NaCl concentration was suitable for efficient enzyme production. The activity of the enzyme was inhibited by all the inhibitors tested. The organism was resistant to antibiotics like bestatin and penicillin. 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 5%. The enzyme retained more than 70% of its activity after 60 min of incubation at 40°C in the presence of various detergents.

1. Introduction

In the living world, each chemical reaction is catalyzed by its own enzyme. Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells. Enzymes exhibit a high specificity, as they are able to discriminate between slightly different substrate molecules. Furthermore they have the ability to operate at moderate temperature, pressure and pH, which makes them attractive catalysts for industrial and household conversion processes. All enzymes which have been purified are protein in nature and may or may not possess a non protein prosthetic group. Jokichi Takamine (1854 - 1922) was the first person to realize the technical possibility of cultivated enzymes and to introduce them to industry. He was mainly concerned with fungal enzymes. Technological process in this field during the last decades has been so great that, for many uses, microbial cultivated enzymes have replaced the animal or plant enzymes.

Enzymes occur in every living cell, hence in all microorganisms. Each single strain of organism produces a large number of enzymes, hydrolyzing, oxidizing or reducing and metabolic in nature. But the absolute and relative amounts of the various individual enzymes produced vary markedly between species and even between strains of the same species. Hence, it is customary to select strains for the commercial production of specific enzymes which have the capacity for producing highest amounts of the particular enzyme desired. Up until less than 10 years ago, commercial bacterial enzymes were produced by surface culture methods. Within the past few years, however, submerged culture methods have come into extensive use. 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).

Industrially available proteolytic enzymes produced by micro organisms are usually mixtures of endopeptidases (proteinases) and exopeptidases.In addition to microbial proteases, the plant proteases bromelin, papin and ficin and the animal proteases pepsin and trypsin, have extensive industrial application. Most proteases are quite specific with regard to which peptide linkages they can split. Hence, it is necessary to select the appropriate protease complex or combination of enzymes for specific applications. With proper selection of enzymes, with appropriate conditions

of time, temperature and pH either limited proteolysis or complete hydrolysis of most proteins to amino acids can be brought about. Proteolytic enzymes are used for tenderizing meats and animal casings for processed meals. Protein hydrolysates for condiments and special diets and for animal feeds are obtained by extensive enzymatic hydrolysis of plant, meat and fish and milk proteins. Pharmaceutical and clinical applications for bacterial proteases (steptokinase-streptodornase) are in debridement of wounds and by injection to relieve inflammation, bruises and blood clots.

Proteases can be produced by all micro organisms; however, only microbes that produce the substantial amount of extracellular proteases have been exploited commercially. Of these, strains of *Bacillus* sp dominate the industrial sector (Gupta *et al.,* 2002*)*.To date the major proportion of commercial proteases are derived from *Bacillus* sp (Joo *et al*., 2002; Manachini *et al*.,1998; Yang *et al*.,2000; Ito *et al*.,1998). The reason for this is their wide temperature, pH tolerance and stability (Genckel and Tari, 2006). *Bacillus* sp mostly produces two kinds of proteases, alkaline and neutral (Rao *et al*., 1998). We believe with Andrade *et al*., 2002, that micro organisms produce large varieties of enzymes, most of which are made in only small amounts and are involved in cellular proteases. Proteolytic enzymes from micro organisms may be located within the cell (intracellular), cell wall associated (periplasmic) or excreted into the media (extracellular) (Kohlmann *et al*., 1991). Extracellular enzymes are usually capable of digesting insoluble nutrient materials such as cellulose, protein and starch and the digested products are transported into the cell where they are used as nutrients for growth (Gibb and Strohl, 1987; Oh *et al*., 2000). 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 *Bacillus* sp is especially important, because the enzymes account for approximately 25% of the world wide enzyme consumption (Gessesse,1997).

The preliminary experiments revealed that *Bacillus coagulans* 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 *Bacillus coagulans* MTCC 492.

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

Bacillus coagulans MTCC 492, a potent protease produce was obtained from IMTECH, Chandigarh 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.

Sterilization

The liquid materials, such as media and distilled water were sterilized in an autoclave at 121° C and 15 pounds per square inch (psi) for 20 minutes. Glasswares were sterilized in hot air oven at 180° C for 2 hours after wrapping in aluminum foil.

Protease production medium

The basal medium used for protease production contained (g/L) beef extract (0.5), peptone (0.3), NaCl (0.5) (Atalo *et al*., 1993). 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 37°C 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 *Bacillus coagulans* was determined using different carbon sources that comprises of potato peel extract, beet root peel extract, carrot peel extract, sweet potato peel extract

Effect of organic nitrogen source

The effect of various organic nitrogen sources was tested on the production of protease by *Bacillus coagulans*. The organic nitrogen sources used were, fish scarp powder, crab shell powder, prawn shell powder, 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

The effect of temperature, pH, NaCl concentration, inhibitors and metal ions were examined here.

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 25-60˚C.

Effect of pH on enzyme stability

The pH stability and activity of enzyme was tested at different pH ranging from 5-9.5 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.

Scaleup of protease

Cultivation of *Bacillus coagulans* 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 37±˚C for 24h with controlled pH at 9.0. 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.

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.

Antibiotic susceptibility of Bacillus coagulans

The invitro antibiotic susceptibility testing of the organism was performed using the standardized disc agar diffusion method described by Baurer *et al.* (1996). Muller Hington agar (MHA) was used as the plating medium. The inoculums were prepared from 24h culture. Sterile cotton tipped buds was dipped into the culture suspension. The swab was then used to streak the entire dried surface of MHA plate. The inoculated plate was incubated for 20 min to allow excess moisture to dry. Then the antibiotic discs were placed at equidistant on the plate. The plates were then incubated at 36°c for about 24h.

The antibiotic discs include, Gentamycin, Kanamycin, Streptomycin, Tetracycline, Chloramphenicol, Eryhromycin, Nalidixicacid, Vancomycin, Bacitracin, Trimethoprim and Penicillin.

3. RESULTS

Protease production in the basal medium

The selected organism (*Bacillus coagulans*) exhibited a large zone of hydrolysis on milk agar plate (Fig 1) and was taken up for optimization of maximum production, scale up and protein characterization.

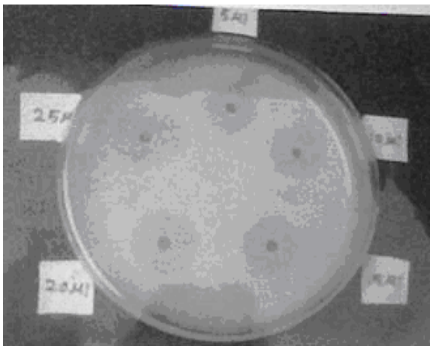

Fig. 1. Zone of inhibition.

The isolate was a gram positive, motile, rod shaped bacterium and strictly aerobic (Table 1). This isolate produced 420 U/ml of protease in the initial production medium.

Table 1. Morphology, physiology and biochemistry of *B. coagulans*

Test	***Bacillus coagulans***
1. Colony morphology	
(a). Grams reaction	+
(b). Cell shape	Rods
(c). Size (μm)	Length: 3-5μm Width: 1.0 μm
(d). Arrangement	Short chain
(e). Endospore	+
(f). Motility	+
(a). Growth at 25-50°C	+
(b). Growth at pH 4-10	+
(c). Growth in NaCl (2-7%)	+
3. Biochemical test	
(a). Indole	-
(b). Methyl red	+
(c). Voges Proskauer	+
(d). Citrate utilization	-
(e). Nitrate reduction	-

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

Bacillus coagulans produced maximum protease in potato peel extract (127 U/ml) followed by beetroot extract (110 U/ml). However, the other

two carbon sources, carrot peel extract (86 U/ml) and sweet potato peel extract (72 U/ml) did not show any remarkable increase in the enzyme production (Figure 2).

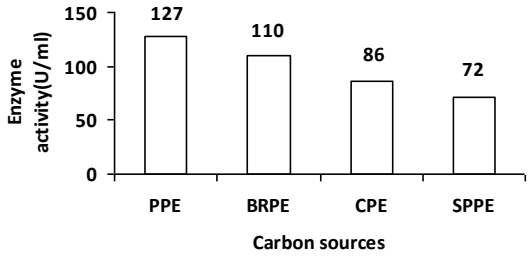

Figure 2. Effect of carbon source on protease activity

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 (227 U/ml) was observed with 1% fish scrap powder (Figure 3). This was followed by prawn shell powder (210 U/ml), crab shell powder (187 U/ml) and skim milk powder (185 U/ml).

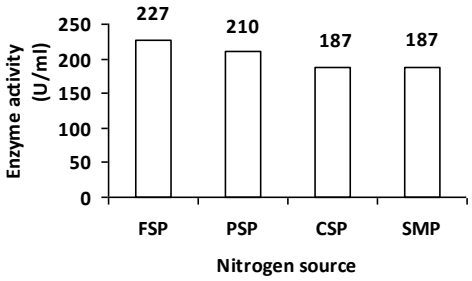

Figure 3. Effect of nitrogen source on protease activity

Enzyme characterization

The effect of temperature, pH, inhibitors, metal ions and NaCl tolerance are studied here.

1. Effect of temperature on protease

The data represented in the (Figure 4) 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. *Bacillus coagulans* exhibited its maximum protease activity between 40˚C and 45˚C. The enzyme lost its activity above 50˚C.

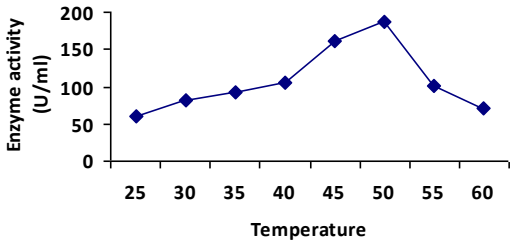

Figure 4. Effect of temperature on protease activity

2. Effect of pH on protease

Data presented in (Figure 5) showed the effect of different pH on the protease activity. Maximum protease activity was exhibited at pH range between 8.5 and 9.

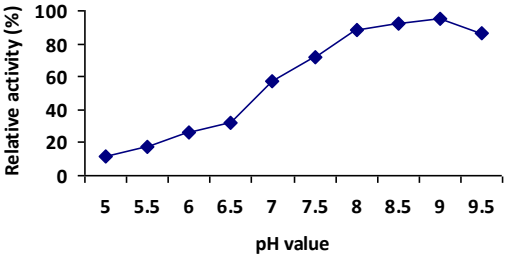

Figure 5. Effect of pH on protease activity

3. NaCl tolerance on protease

The alkaline protease production by *Bacillus coagulans* was studied within the range of 1-7% Nacl concentration, but maximum production was observed at 2% concentration (Figure 6).

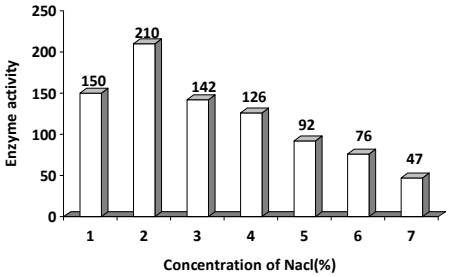

Figure 6. Effect of NaCl tolerance

4. Effect of inhibitors on protease

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 (Table2).

Table 2. Effect of inhibitors on protease activity

Inhibitor	Relative activity (%)
1. Phenyl Methyl Sulphonyl Flouride (PMSF)	NIL
2. β-Mercapto Ethanol	90
3. Ethelene Di Amine Tetra Acetate (EDTA)	64
4. Bestatin	10
5. Chymostatin	05

5. Effect of metal ions on protease

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 (Table 3). 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.

Table 3: Effect of metal ions on protease activity

Metal ions	Relative activity (%)
1. $CaCl_2$	105
2. $MgCl_2$	96.3
3. $CuSO_4$	68
4. Urea	63
5. $ZnCl_2$	21

Growth curve studies on Bacillus coagulans

During this study, the organism was found to be fast growing. The maximum proteolytic activity (256 U/ml) was observed after 24h of growth where it reached stationary phase. The alkaline protease activity was stable even after 48 hours of growth (Figure 7).

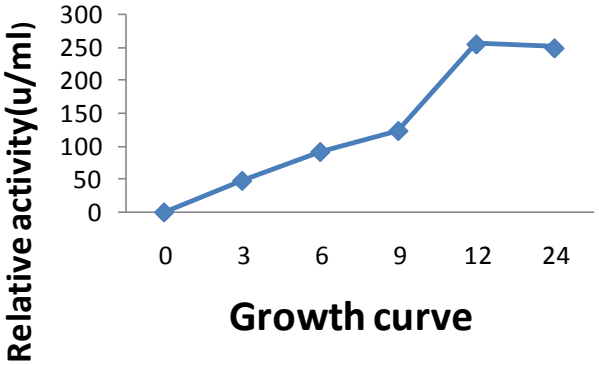

Figure 7. Growth curve studies on *Bacillus coagulans*

Scale up of protease

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 8). Thereafter, there was a decline in the protease production in the bioreactor.

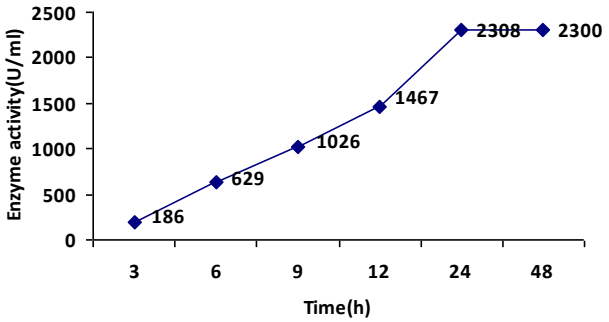

Figure 8. Scaleup of protease

Partial purification of the enzyme

The produced enzyme was partially purified by ammonium sulphate precipitation method (Figure 9). The crude enzyme was found to be 60% pure. Further purification can be done by various chromatographic methods to obtain 100% pure enzyme.

Figure 9. Partial purification of enzyme

Molecular weight determination

Electrophoresis on SDS-PAGE revealed a single band of protein corresponding to a molecular weight of 36KDa (Figure 10).

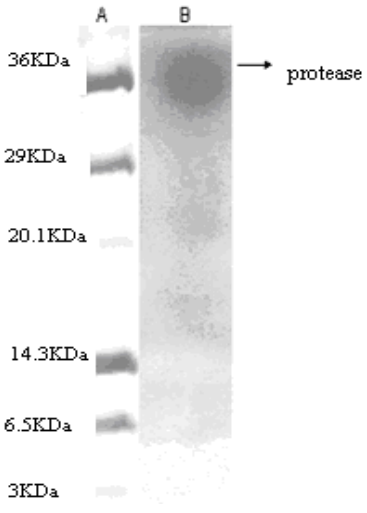

Figure 10. SDS-PAGE Analysis

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 11).

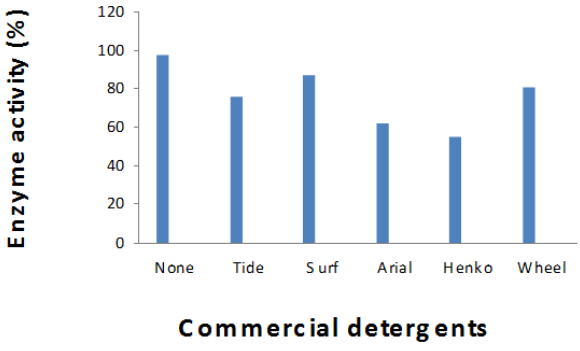

Figure 11. Compatibility with detergents

Antibiotic susceptibility of Bacillus coagulans

Bacillus coagulans was assayed for its susceptibility to 12 antibiotics. The organism was found to be susceptible to almost 10 antibiotics used. But it was resistant to bacitracin and penicillin. The organism was resistant to bacitracin because, it is a product from Bacillus sp. (Figure 12).

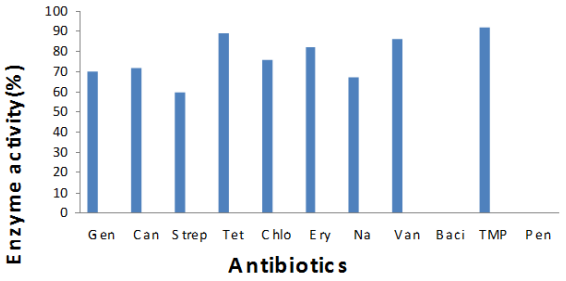

Figure 12. Antibiotic susceptibility test

4. DISCUSSION

The selected organism (*Bacillus coagulans* MTCC492) exhibited a large zone of hydrolysis on milk agar plate (figure 1) and was taken up for optimization of maximum production, scale up and protein characterization. The isolate was gram positive, motile, rod shaped bacterium (table 1).

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, *Bacillus coagulans* produced maximum protease in potato peel extract (127 U/ml) followed by the other extracts (Figure 2). 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 Bacillus Sp. Sourabh *et al.,* 2007, used soybean as the substrate for the production of alkaline protease by Bacillus Sp. 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 227 U/ml (figure 3). 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. There was no doubt that temperature was an important environmental factor that affects the growth of the microorganisms as well as production of enzymes. *Bacillus coagulans* exhibited its maximum protease activity between 40-45˚C (figure 4). 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 horikoshii* respectively (Fujiwara *et al*., 1993).

The organism produced maximum protease at a pH ranged 8.5-9 (figure 5).This is in complete contrast with the findings of Remeikaite, 1979; Do Nascimento and Martin, 2004 and Wang *et al*. According to their findings the optimum pH for the maximum protease production was 7.5 for *Bacillus subtilis*, 7.2 for *Bacillus subtilis* k11 and *Bacillus licheniformis*, 6.9 for *Bacillus* p. SMIA-2 and 7 for *Bacillus* Sp. TKU004.

The alkaline protease production by *Bacillus coagulans* was studied within the range of 1-7%. NaCl concentration and the maximum production were observed at 2% concentration (figure 6). 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 (table 2). 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 (table 3). 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 *Bacillus 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, *Bacillus coagulans* 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 2308 U/ml in 24h (figure 8). There after, 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 (figure 9). 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 *Bacillus brevis geltinoamylolyticus* (Ammar *et al*., 2003). More over, 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 36KDa (figure 10). 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.

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 11). Phadatare *et al*. (1993) reported high activity of alkaline protease from *conidiobolus coronatus* which showed compatibility at 50˚C in the presence of 25mM $cacl_2$ with a variety of commercial detergents.

Bacillus coagulans was assayed for its susceptibility to 12 antibiotics. The organism was found to be susceptible to almost 10 antibiotics used. But it was resistant to bacitracin and penicillin. The organism was resistant to bacitracin because, it is a product from Bacillus Sp. In contrast to this result Green *et al*., 1999, observed that a probiotic *Bacillus subtilis* was sensitive to penicillin, tetracycline, chloramphenicol and erythromycin.

In summary, *Bacillus coagulans* MTCC 492, 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 9 and 45˚C respectively. The organism was found to be resistant to antibiotics namely bacitracin and penicillin. The enzyme was of molecular weight 36KDa and 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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