

Full Length Research Paper

Purification and characterization of protease from *Bacillus cereus* SU12 isolated from oyster *Saccostrea cucullata*

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Accepted 16 September, 2013

Protease-producing bacterium *Bacillus cereus* SU12 was isolated from oyster *Saccostrea cucullata*. Fifteen strains of bacteria were isolated from oyster *S. cucullata* and screened for secretion of protease on casein agar plates. Among them, SU12 isolate was selected due to its high enzyme production capacity and was identified as *B. cereus* SU12 on the basis of its morphological, biochemical and 16S rDNA properties. Media and cultivation conditions were studied to optimize bacterial growth and protease production which includes different carbon and nitrogen sources, in addition to different factors such as incubation time, pH, temperature, NaCl concentrations. At pH 7, temperature 40°C and 2.5% NaCl concentration, carbon source such as starch and beef extract as nitrogen source, the protease activity was maximum. Extracellular protease was isolated, purified to 8.73 fold by diethyl aminoethyl (DEAE) ion-exchange chromatography and its specific activity was determined to be 886.56 U/mg and the sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) showed a single band for the purified enzyme, with an apparent molecular weight of 66 kDa. These findings suggest that the scope for the use of *B. cereus* SU12 strain as suited organism for the industrial production of the extracellular protease enzyme.

Key words: Protease, *Bacillus cereus* SU12, Oyster, diethyl aminoethyl (DEAE) ion-exchange chromatography, optimization.

INTRODUCTION

With recent advent of biotechnology, there has been a growing interest and demand for enzymes with novel properties. Considerable efforts have been directed towards the selection of microorganisms producing enzymes with new physiological properties and tolerance to extreme conditions used in industrial processes such as temperature, salts, pH among others. More than 75% of industrial enzymes are observed as hydrolases (Leiola

et al., 2001). Proteases constitute one of the most important groups of hydrolytic enzymes which act upon native proteins to disintegrate them into small peptides and amino acids (Nascimento and Martins, 2004).

Marine microorganisms have recently emerged as rich sources for the isolation of industrial enzymes (Adinarayana and Ellaiah, 2002). Researchers throughout the world target marine niches for enzymes as they

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Abbreviations: PCR, Polymerase chain reaction; BLAST, Basic Local Alignment Search Tool; NCBI, National Centre for Biotechnological Information; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

display novel characteristics such as tolerance to extreme pH, temperature, salinity among others. In marine habitats, there are relatively high proportions of proteolytic bacteria as compared to freshwater or soil habitats (Atlas and Bartha, 1981). Marine microbes are now being looked upon as a potential source of various compounds: pharmaceutical, nutritional supplements, agrochemicals, cosmetics and enzymes (Vignesh et al., 2011; Baharum et al., 2010). The basic characteristics of the enzymes derived from the marine sources differ from the terrestrial sources due to their natural habitat. Marine microbial enzymes are reported to be more stable and active than those originating from plant and animal sources since they possess almost all characteristics desired for their biotechnological applications (Bull et al., 2000).

Proteases are the most important industrial enzymes accounting for about 50% of the total industrial enzymes (Rao et al., 1998). They are the class of enzymes which occupy a pivotal position due to their wide-spread application in detergent, pharmaceutical, photography, leather, laundry, food and agricultural industries. These enzymes are also used in baking, brewing, meat tenderization, peptide synthesis, medical diagnosis, cheese making, and bioremediation process and as treatment against inflammation and virulent wounds (Anwar and Saleemuddin, 1998; Gupta et al., 2002). Microbial enzymes find a broad spectrum of application due to their broad biochemical diversity, ease of mass culture that is, rapid growth in limited space and genetic manipulation (Adsul et al., 2007; Das and Prasad, 2010). Among the various proteases, bacterial proteases are most significant compared to animal and fungal proteases. Amongst bacteria, *Bacillus* species are specialized producers of extracellular proteases. Most of the commercial alkaline proteases were isolated from *Bacillus* species (Priest, 1977).

Bacillus cereus is one of the most widely used bacteria for the production of specific chemicals and industrial enzymes and also a major source of protease enzyme. Bioactive potential of bivalve species was rarely studied. Oysters are edible sea food. It is abundant resource in marine environment. However, there is no study on isolation of microbial enzyme from oyster. Therefore, the present study was focused on the isolation, identification and characterization of protease producing bacteria from oyster *Saccostrea cucullata*. We also report here the purification and characterization of protease from *B. cereus* SU12 isolated from gut of oyster *S. cucullata*.

MATERIALS AND METHODS

Sample collection

Oysters (*S. cucullata*) were collected from the Mandapam coast (Lat. 09° 17.417'N; Long. 079° 08.558'E). Then the oysters were transported to the laboratory in an ice box within 2-3 h after collection. Further, the animals were cleaned, opened aseptically

and shucked as followed by Hunt et al. (1984).

Isolation and characterization of protease producing bacteria

Gut is important tissue for isolation of potential microbes in many marine species. Oyster gut tissue was weighed in a sterile beaker, and ground in mortar and pestle. Then the sample was serially diluted (10^{-2} to 10^{-9}) and spread onto nutrient agar media. Plates were incubated at 28°C for 48 h.

The bacteria were sub-cultured on casein agar and the isolates, which produced clear zone on casein agar after 24 h incubation were maintained on nutrient agar plates. The potential isolate showed clear zone of inhibition than other strains on casein agar was retained for this study. The isolated bacterial strain was identified on the basis of their morphological, cultural and biochemical characteristics. The obtained data were compared with standard description provided in Bergey's manual of determinative bacteriology (Bergey and Holt, 1994).

16S rDNA sequencing and analysis

Genomic DNA was extracted from the cells of an 18 h culture using Wizard® genomic DNA Purification kit (Promega). The 16S rDNA sequences were amplified by polymerase chain reaction (PCR) using universal primers of 27f (5'-AGAGTTTGATCCTGTGCTCAG-3') and 1492r (5'-GACTTACCAGGGTATCTAATCC-3'). PCR products were electrophoresed on 1% agarose gel and documented (Kumaran et al., 2010). The PCR product was purified by using PCR purification kit (Genei, Bangalore, India). The nucleotide sequences of the PCR product was determined by using the automated DNA sequencer with forward and reverse primers (Bio-serve Bio Technologies Pvt. Limited Hyderabad, India). Sequence comparison with the databases was performed using Basic Alignment Search Tool (BLAST) through the National Centre for Biotechnological Information (NCBI), USA, server. The isolate was identified as *B. cereus*.

Nucleotide accession number

The obtained nucleotide sequence of *B. cereus* strain was submitted in GenBank database and the assigned accession number is JX080200.

Phylogenetic analysis

Sequence similarity search was made for the 16S rDNA sequence of the identified strain by applying their sequence to BLAST in NCBI (USA). Phylogenetic analysis was performed using the software package Molecular Evolutionary Genetics Analysis (MEGA) version 4 (Kumar and Tamura, 2001) after multiple alignment of data by CLUSTAL X (Thompson et al., 1997) and a phylogenetic tree was reconstructed by using the neighbor-joining method of Saitou and Nei (1987).

Production of protease

Production of protease from *B. cereus* SU12 was carried out in a medium containing 0.5% casein (w/v), 0.2% starch (w/v), 0.2% KH_2PO_4 (w/v), 0.2% KNO_3 (w/v), 0.5% NaCl (w/v), 0.005% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (w/v), 0.002% CaCO_3 (w/v), 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (w/v) with pH adjusted to 7.4. The fermentation medium was maintained at 37°C for 36 h at 200 rpm in a shaking incubator. At the end of the fermentation period, the broth was centrifuged at 10,000 rpm for 15

min at 4°C. The clear supernatant was recovered as crude enzyme preparation and subjected to purification for further studies.

Optimization of culture conditions

The strain SU12 was subjected to different culture conditions to derive the optimum conditions for protease production. Protease production was estimated at various temperatures, pH, sodium chloride, carbon sources and nitrogen sources. Experiment was conducted in triplicate at various temperatures viz. 35, 40, 45, 50, 55 and 60°C to study their effect on protease production. Average of triplicate values has been plotted. Different pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) of the casein broth was tested to study their effect on protease production. All the experiments were carried out in triplicate and average values has been plotted.

The effect of various concentrations of sodium chloride (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%) on protease production was studied by changing the ratio of volume of sodium chloride solution to the casein broth and the flasks were incubated in triplicate and the average values were plotted. Casein broth was used for studying the effect of various carbon compounds viz. starch, mannitol, glucose, dextrose, xylose, and arabinose. The broth was distributed into various flasks and 1% of each carbon source was added before inoculating of the strain (SU12) and was incubated at the optimum pH, temperature and NaCl. The experiments were conducted in triplicate and the average values were plotted.

Casein broth was used for studying the effect of various nitrogen compounds viz. beef extract, peptone, yeast extract, potassium nitrate (KNO₃) and ammonium sulfate. The broth was distributed into various flasks and 1% of each nitrogen source was added before strain inoculation and was incubated at standardized parameters. The experiments were conducted in triplicate and the average values were plotted.

Incubation period

The effect of incubation periods on protease production was studied. For this, 50 ml of casein broth containing optimized carbon and nitrogen sources was taken in each 100 ml conical flask. After autoclaving, the flasks were inoculated with equal quantity of inoculums and incubated at optimal conditions (temperature, pH) for 6, 12, 18, 24, 30, 36 h. The culture filtrates were collected and used for enzyme activity. The experiments were conducted in triplicate and the average values were plotted.

Enzyme purification

The clear supernatant (crude protease) was precipitated with different concentrations of ammonium sulphate that is, from 10 - 80% saturation. The precipitated protein was dissolved in 20 mM potassium phosphate buffer and dialyzed against same buffer. Further purification was carried out in ion exchange chromatography (DEAE-Cellulose). The dialyzed protein was applied to a DEAE-cellulose column (2.5 × 70 cm), pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.0). After washing the column with 3 volume of equilibration buffer, bound proteins were eluted stepwise by using phosphate buffers in increasing molarity and decreasing pH values at room temperature. The flow rate was adjusted to 0.5 ml/min and fractions (1 ml each) were collected. The fractions showing high enzyme activity were pooled and lyophilized.

Determination of protein

Protein concentration was measured following Lowry et al. (1951)

method. Bovine serum albumin (BSA) was used as the standard.

Protease assay

Protease activity was determined following the method of Kembhavi et al. (1993) and 2.0% casein was used as substrate. One milliliter (1 ml) diluted enzyme solution was mixed with 1 ml of 2.0% casein in 50 mM Tris-HCl (pH 8.5) and incubated at 30°C for 10 min. After incubation, the reaction was stopped by the addition of 2 ml of 0.4 M trichloroacetic acid. Then, the precipitate was removed by centrifugation at 10,000 rpm for 10 min, and 1 ml of supernatant was neutralized with 5 ml of 0.4 M sodium carbonate and incubated with 1 ml of 1 N Folin Ciocalteu's reagent solution at 40°C for 20 min. Subsequently the absorbance at 660 nm was measured. The enzyme activity was calculated from standard curve of L-tyrosine. One unit of activity was defined as the amount of enzyme that liberated 1 µg of tyrosine per milliliter of reaction mixture per minute.

Molecular weight determination of protease in SDS-PAGE

The molecular weight of the protease was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed according to Laemmli (1970) using 4% stacking gel and 12% resolving gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. The molecular weight of the protease was determined by comparison with the migration distances of standard marker proteins consisting of phosphorylase B (97.4 kDa), albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.3 kDa).

RESULTS AND DISCUSSION

Protease is an industrially important enzyme having wider applications in pharmaceutical, leather, laundry, food and waste processing industries. Industrial enzyme production would be effective only if the organism and the target enzyme are capable of tolerating different variables of the production processes. Primary screening for protease producing bacterial strains was done in casein agar medium based on zone formation. The bacterial isolates which formed zone around the colonies were considered to be protease positive strain. The clear zone formation may be attributed to the hydrolysis of casein. Fifteen (15) bacterial colonies (SU1 to SU15) secreting protease were isolated. The isolates were purified through repeated streaking onto casein agar plates. Among the isolates, the best (SU12) was selected based on highest zone formation (casein hydrolysis) on casein agar (Figure 1). The potential strain was maintained on agar slants and stored at 4°C.

Identification of the protease producing strain

Microscopic observation of isolate SU12 showed motile, Gram positive rod shaped bacterium; the bacterium grew aerobically and formed white colonies. The morphological and biochemical characteristics are presented in Table 1.



Figure 1. Proteolytic activity of *B. cereus* SU12.

Table 1. Characteristics of *Bacillus cereus* SU12.

| Morphological and biochemical tests | <i>Bacillus cereus</i> SU12 |
|-------------------------------------|------------------------------|
| Morphological identification | Small, white, dried colonies |
| Gram's staining | Gram positive, rod |
| Indole production | - |
| Methyl red test | - |
| Voges - Proskauer test | + |
| Citrate utilization test | + |
| Starch hydrolysis | + |
| Casein hydrolysis | + |
| Urease test | - |
| Motility | + |
| Carbohydrate fermentation test | |
| a) D-glucose | + |
| b) Mannitol | - |
| c) Lactose | - |
| d) Sucrose | + |

+, Positive results; -, negative results.

Based on Bergey's manual of determinative bacteriology the phenotypic characteristics of isolate (SU12), belongs to the genus *Bacillus*.

Genomic DNA was isolated from the strain SU12 and its quality was checked by loading in 1% agarose gel with the DNA marker which showed the intact DNA. 16S rDNA of the strain SU12 was amplified through PCR which shows the molecular weight of 1.472 kb corresponding to that of the DNA ladder in 1% agarose gel (Figure 2). The amplified product was sequenced (Figure 3). The obtained 16S rDNA gene sequence (1472 bp) of the strain SU12 was preliminary compared with previously obtained sequen-

ces of *Bacillus* sp deposited in GenBank (NCBI) and it indicated that this organism is phylogenetically related to the members of the genus *Bacillus*. The phylogenetic tree of 16S rDNA sequences was constructed by using the three valid representative species of the genus *Bacillus* to know the relationship of the strain SU12 and the BLAST result showed that they appeared close match in neighboring tree. The sequence of *B. cereus* (HM771668.1) served as outside reference of operational taxonomic unit (Figure 4). The species *B. cereus* (AB682146.1), *Bacillus thuringiensis* (EU429660.1), *Bacillus* sp (GQ407198.1) has the closest sequence similarity of 99%.

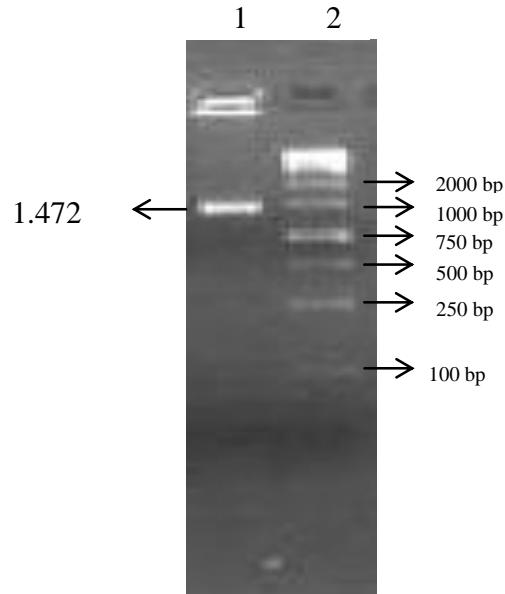


Figure 2. Amplified product of 16S rDNA of strain SU12. Lane 1, Strain SU12; Lane 2, DNA marker.

ORIGIN

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1 accggaggcg cgccccgctt aaaacatggc aagtcggagc gaatggatta agagcttgct
61 cttatgaagt tagcggcgga cgggtgagta acacgtgggt aacctgcccc taagactggg
121 ataactccgg gaaaccgggg ctaataccgg ataacatttt gaaccgcatg gttcgaattt
181 gaaaggcggc ttcggctgtc acttatggat ggaccgcgct cgcattagct agttggtagg
241 gtaacggctc accaaggcaa cgatgcgtag ccgacctgag agggatgatc gccacactgg
301 gactgagaca cggcccagac tcctacggga ggcagcagta gggaatcttc cgcaatggac
361 gaaagtctga cggagcaacg ccgcgtgagt gatgaaggct ttcgggtcgt aaaactctgt
421 tgtaggggaa gaacaagtgc tagtgaata agctggcacc ttgacggtag ctaaccagaa
481 agccacggct aactacgtgc cagcagccgc ggtaatacgt aggtggcaag cgttatccgg
541 aattattggg cgtaaagcgc gcgcaggtgg tttcttaagt ctgatgtgaa agcccacggc
601 tcaaccgtgg agggtcattg gaaactggga gacttgagtg cagaagagga aagtggaatt
661 ccatgtgtag cggtgaaatg cgtagagata tggaggaaca ccagtggcga aggcgacttt
721 ctggtctgta actgacactg aggcgcgaaa gcgtggggag caaacaggat tagataccct
781 ggtagtccac gccgtaaacg atgagtgcta agtggttagag ggtttccgcc ctttagtgct
841 gaagttaacg cattaagcac tccgcctggg gagtacggcc gcaaggctga aactcaaagg
901 aattgacagg ggcccgcaca agcgggtggag catgtggttt aattcgaagc aacgcgaaga
961 accttaccag tcttgacatc ctctgacaac cctagagata gggcttctcc ttcgggagca
1021 gagtgacagg tgggtgcatg ttgtcgtcag ctcgtgctgt gagatgttgg gttaagtccc
1081 gcaacgagcg caacccttga tcttagttgc catcatttag ttgggcactc taaggtagct
1141 gccggtgaca aaccggagga aggtggggat gacgtcaaat catcatgccc cttatgacct
1201 gggctacaca cgtgctacaa tggacggtag aaagagctgc aagaccgcca ggtggagcta
1261 atctcataaa accgttctca gttcggattg taggctgcaa ctcgcctaca tgaagctgga
1321 atcgctagta atcgcgatc agcatgccgc ggtgaatac tccccgggcc ttgtacacac
1381 cgcccgctac accacgagag tttgtaacac ccgaagtcgg tggggtaacc tttttggagc
1441 cagcccgccc taaatggggg aacaagaatt tt

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Figure 3. 16S rDNA sequence of the strain SU12.

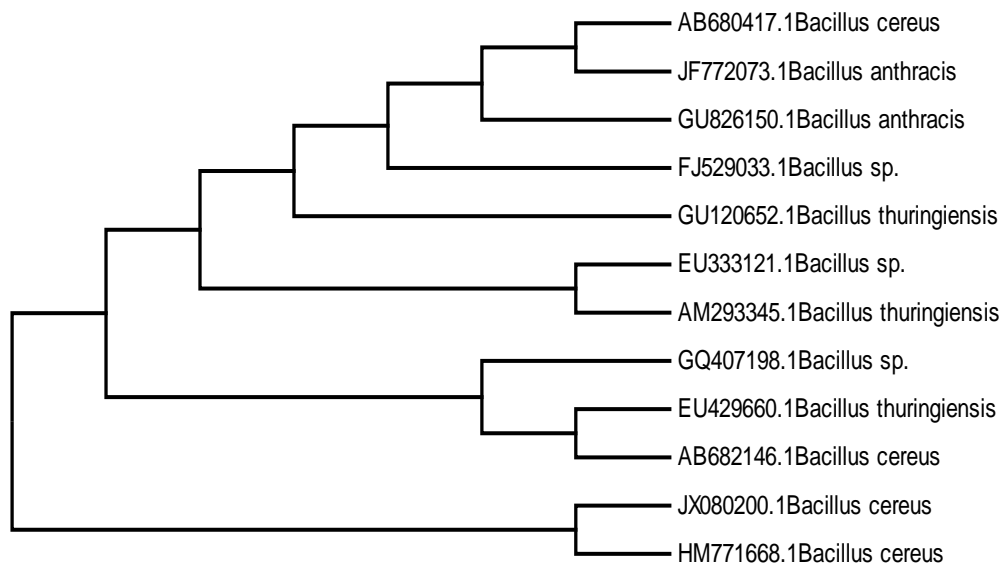


Figure 4. Neighbor-joining tree based on 16S rDNA sequences, showing relationship between the strain SU12 (1472 nucleotides) and 11 bacterial species.

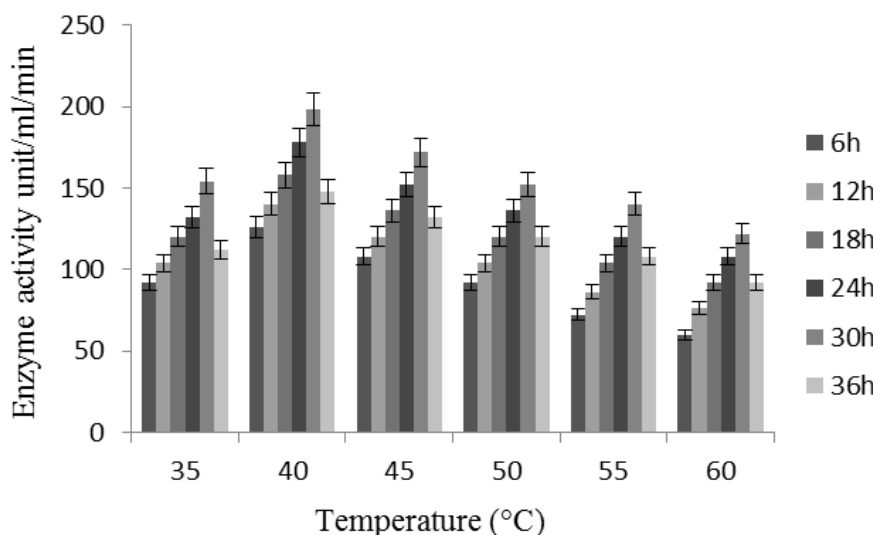


Figure 5. Effects of temperature on protease activity of *B. cereus* SU12. Each value is the mean \pm SD of triplicate measurements.

Optimization of culture conditions

Factors such as temperature, pH, sodium chloride concentration, different carbon sources and nitrogen sources which may influence the secretion of protease enzyme were optimized for maximum protease production and activity by *B. cereus* SU12. Standardization of culture condition was carried out in one parameter at one time and the standardized values were used for subsequent experiments.

Effects of temperature on protease activity

The activity of protease at different temperatures was determined by incubating the reaction mixture at temperatures ranging from 35 to 60°C for different time intervals before determining protease activity. Experiments were carried out in triplicate and the average values were reported. Maximum enzyme production (198 \pm 0.3 unit/ml) was observed at 40°C (Figure 5), and relatively minimum level (122 \pm 0.2 unit/ml) was observed

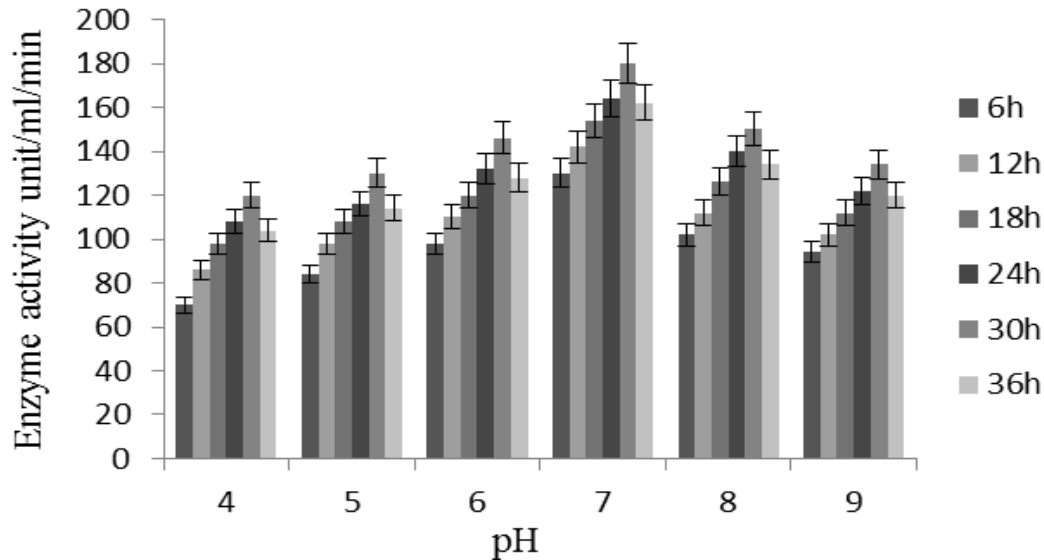


Figure 6. Effects of pH on protease activity of *B. cereus* SU12. Each value is the mean \pm SD of triplicate measurements.

at 60°C for 30 h of incubation. Similar results were reported for other *Bacillus* proteases. For example, the optimum temperature for protease from *Bacillus amovivorus* (Sharmin et al., 2005), *Bacillus fastidiosus* (Shumi et al., 2004) and *Pseudomonas fluorescens* CM1₁₂ (Al-Saleh and Zahran, 1997) was 37°C. The present study recorded 40°C as optimal, which is in agreement with earlier findings of VijayAnand et al. (2010) and Fulzele et al. (2011), optimum temperature for protease was 40°C. El-Safey and Abdul-Raouf (2004) reported the same findings in production, purification and characterization of protease enzyme from *Bacillus subtilis*. Related studies also reported that protease production by *Bacillus laterosporus* was best at 40°C (Usharani and Muthuraj, 2010).

Effects of pH on protease activity

The effect of pH on protease activity was determined in different pH values ranging from 4.0 to 9.0. Reaction mixtures were incubated at different time intervals and the activity of the protease was measured. The result showed that the enzyme activity was maximum (180 \pm 0.06 unit/ml) at pH 7 and minimum (120 \pm 0.02 unit/ml) at pH 4 (Figure 6). Similar results were obtained for the optimum pH for enzymatic activity of other *Bacillus* sp: pH 7.5 for *B. subtilis* ITBCCB 148 (Yandri et al., 2008), *Bacillus* sp. HS08 (Huang et al., 2006) and *Bacillus* sp. S17110 (Jung et al., 2007). In another study, Maal et al. (2009) reported, pH 7 is optimal for the maximum production of alkaline protease from *B. cereus* and *Bacillus polymixa*. Several other findings also described pH 7 is optimal for the maximum protease

production (Gouda, 2006; Fulzele et al., 2011; Vidyasagar et al., 2006).

Sodium chloride

As the organism was isolated from marine environment, the use of varying percentage of NaCl in the production medium was of interest. The results confirmed that the enzyme activity was at its best (190 \pm 0.04 unit/ml) when the concentration of NaCl was 2.5% (Figure 7). Similarly, Manivasagan et al. (2010) observed the maximum protease activity at 2% sodium chloride concentration by the actinobacteria isolated from sediment samples. Vonothini et al. (2008) also reported maximum protease activity of actinomycete Strain, PS-18A at 3% sodium chloride concentration.

Carbon compounds

Different carbon sources were used in the production medium for determining the highest yield of enzyme production. The highest enzyme activity (184 \pm 0.5 unit/ml) was obtained when starch was used as the carbon source (Figure 8), while the activity was minimum (116 \pm 0.4 unit/ml) with arabinose. This is in agreement with the previous reports which showed that starch and glucose caused high level of enzyme expression in *Bacillus* sp. and *B. cereus* strain 146, respectively (Mahmood et al., 2000; Shafee et al., 2005). Similarly, some research groups have shown an increase in protease production by *Bacillus* sp. in the presence of starch (Ferrero et al., 1996; Gusek et al., 1988; Hubner et

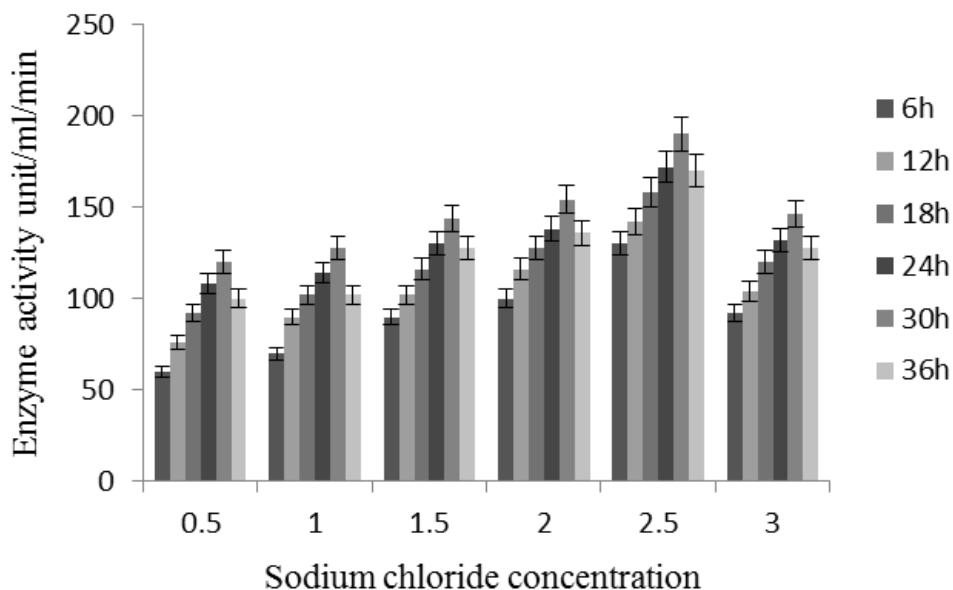


Figure 7. Effects of sodium chloride on protease activity of *B. cereus* SU12. Each value is the mean \pm SD of triplicate measurements.

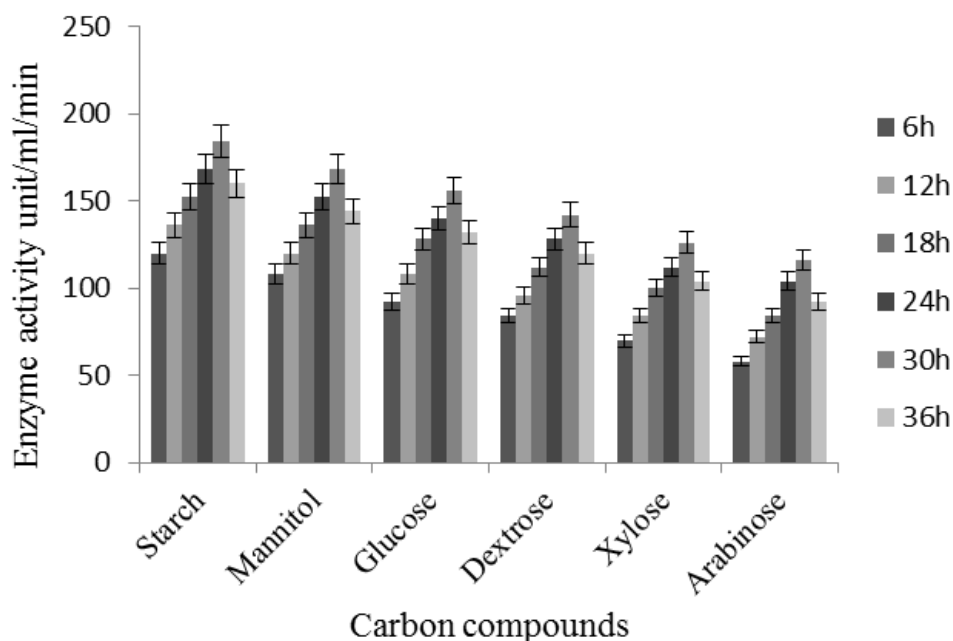


Figure 8. Effects of carbon compounds on protease activity of *B. cereus* SU12. Each value is the mean \pm SD of triplicate measurements.

al., 1993; Puri et al., 2002). Studies on alkaline proteases reported that the addition of starch to the culture medium induced enzymes synthesis (Chauhan and Gupta, 2004; Fang et al., 2001). However, other works reported better protease synthesis in the presence of glucose as carbon source (Ferrero et al., 1996; Mehrotra et al., 1999).

Nitrogen compounds

Culture broth was used for studying the effect of various nitrogen compounds viz. beef extract, peptone, yeast extract, potassium nitrate (KNO_3) and ammonium sulfate

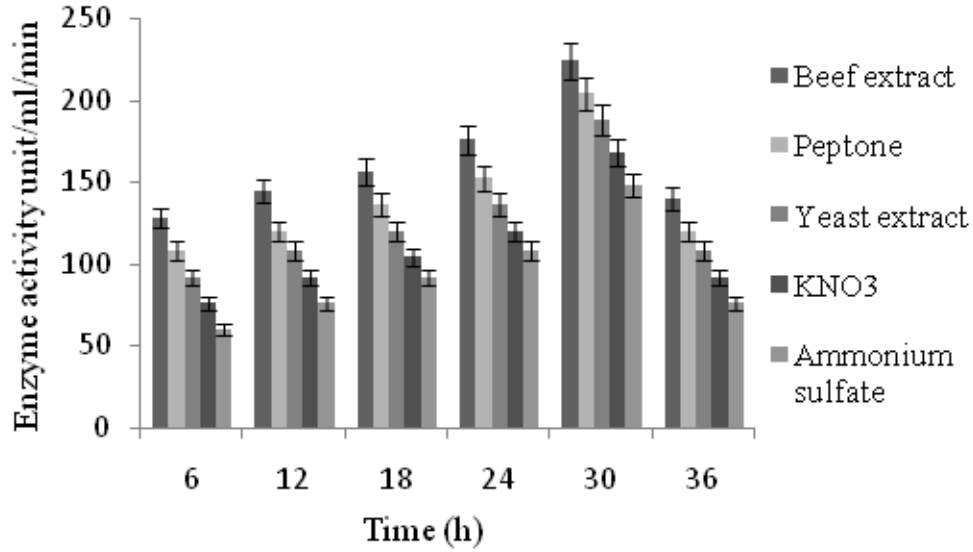


Figure 9. Effects of nitrogen compounds on protease activity of *B. cereus* SU12. Each value is the mean±SD of triplicate measurements.

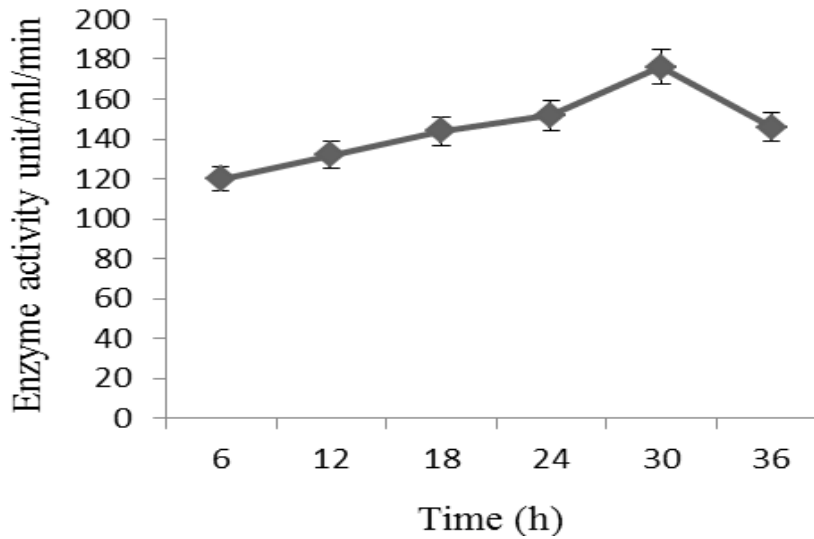


Figure 10. Effects of incubation period on protease activity of *B. cereus* SU12. Each value is the mean±SD of triplicate measurements.

on enzyme activity. Of the different nitrogen sources, the test strain showed maximum enzyme activity (224±0.05 unit/ml) in the presence of beef extract (Figure 9). Similarly Vonothini et al. (2008) and Manivasagan et al. (2010) also observed maximum enzyme activity in the beef extract as nitrogen source in actinobacteria. Researchers have reported soybean meal as the best nitrogen source for protease production from *Bacillus* sp (Masse and Tilburg, 1983; Bhunia et al., 2010; Hubner et al., 1993; Puri et al., 2002). Likewise, other reports demonstrated peptone as the better nitrogen source for

protease production from *Bacillus* sp (Das and Prasad, 2010; Adinarayana and Ellaiah, 2002).

Effects of incubation period on protease activity

Since microorganisms show considerable variation at different incubation period, it is very essential to detect the optimum incubation time at which an organism shows the highest enzyme activity. Figure 10 shows maximum level of protease production (176±0.05 unit/ml) at 30 h

Table 2. Effects of incubation period on the biomass yield of *B. cereus* SU12.

| Incubation periods (h) | Biomass yield (absorbance at 600 nm) |
|------------------------|--------------------------------------|
| 6 | 0.65 |
| 12 | 0.752 |
| 18 | 0.856 |
| 24 | 0.915 |
| 30 | 0.974 |
| 36 | 1.143 |

Table 3. Optimal levels of growth parameters and sources for protease production by the strain SU12.

| S/N | Parameter | Optimal value/sources |
|-----|--------------------|-----------------------|
| 1 | Incubation time | 30 h |
| 2 | Temperature (°C) | 40 |
| 3 | pH | 7.0 |
| 4 | NaCl concentration | 2.5% |
| 5 | Carbon source | Starch |
| 6 | Nitrogen source | Beef extract |

Table 4. Summary of the protease purification from *B. cereus* SU12.

| Purification step | Volume (ml) | Total activity (U/ml) | Total protein (mg) | Specific activity (U/mg) | Purification fold | Yield (%) |
|--|-------------|-----------------------|--------------------|--------------------------|-------------------|-----------|
| Crude extract | 100 | 4579.45 | 137.8 | 33.23 | 1 | 100 |
| Ammonium sulfate precipitation (80% saturation and dialysis) | 25 | 2975.32 | 29.3 | 101.55 | 3.05 | 64.97 |
| DEAE-cellulose chromatography | 5 | 1125.93 | 1.27 | 886.56 | 8.73 | 37.84 |

and minimum level of enzyme production (120 ± 0.03 unit/ml) at 6 h of incubation period. Maximum production of proteases with 48 h of incubation by bacteria was reported by Hoshino et al. (1997) and Shumi et al. (2004). In the present study, 30 h of incubation is suitable time for maximum production of proteases. The highest biomass yield was recorded at 36 h of incubation period (Table 2). Optimal levels of the growth parameters observed in the present study for the production of protease with respect to the potential bacterial strain SU12 are shown in Table 3.

Purification of protease enzyme

The protease from *B. cereus* SU12 was purified with ammonium sulfate fractionation, followed by DEAE-ion exchange chromatography. Results of protease purification are summarized in Table 4. The specific activity after the

80% saturation with ammonium sulfate was 101.55 U/mg protein and the purification was 3.05 fold. The purified enzyme of strain SU12 after DEAE cellulose chromatography exhibited a specific activity of 886.56 U/mg protein with 8.73 fold purification.

Molecular weight determination in SDS-PAGE

Molecular weight of the protease enzyme was determined by SDS-PAGE (Figure 11). Single protein band was observed when stained with Coomassie Brilliant Blue and it clearly indicated the purity of the protein. The molecular weight of the purified enzyme was 66 kDa. A variety of molecular mass for proteases from other *Bacillus* species had been reported: 30.9 kDa thermophilic *Bacillus* strain HS08 (Huang et al., 2006); 27.0 kDa *Bacillus megaterium* (Reungsang et al., 2006); 75.0 kDa *Bacillus sp.* S17110 (Jung et al., 2007); 34.0 kDa *B. thuringiensis* (Kunitate et

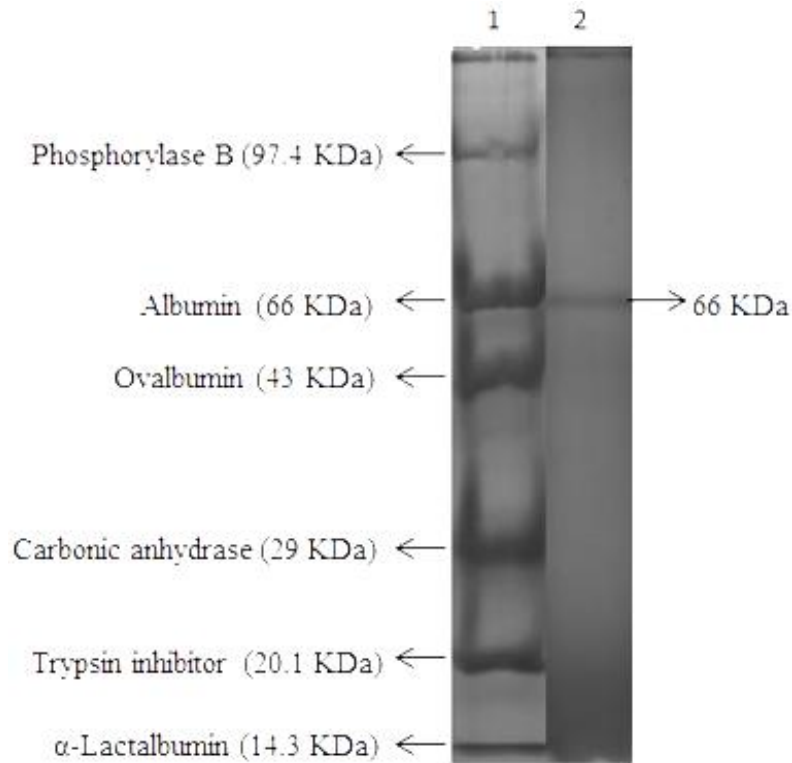


Figure 11. SDS-PAGE of the purified enzyme: Lane1: Molecular weight markers; Lane2: Protease enzyme.

al., 1989); 38.0 kDa *B. cereus* KCTC 3674 (Kim et al., 2001); 15.0 kDa *B. subtilis* PE-11 (Adinarayana et al., 2003); 34.0 kDa *B. cereus* BG1 (Ghorbel-Frikha et al., 2005); 66.2 kDa, 31.0 kDa and 20.1 kDa *Bacillus licheniformis* strains BLP1, BLP2 and BLP3, respectively (Cheng et al., 2006).

Conclusions

From the present study, it is concluded that the identified species, *B. cereus* SU12 isolated from the *S. cucullata* possesses good protease activity. The study has also standardized the growth parameters of bacteria for the maximum enzyme production, which can be effectively used in the large scale production of protease for commercial purposes. The optimum temperature and pH were determined as 40°C and 7.0 and best carbon and nitrogen sources were starch and beef extract. This information has enabled the formulation of media composition for maximum protease production by this organism. The protease was purified by ammonium sulfate precipitation and DEAE-cellulose chromatography. The molecular weight of protease was found to be 66 kDa. The purified protease will be used for the various purposes in detergent industries, food industries and pharmaceutical industries.

ACKNOWLEDGEMENTS

The authors would like to thank Ministry Of Earth Sciences for financial support, under the “Drugs from sea” programme and the authors are grateful to the authorities of Annamalai University for providing the necessary facilities.

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