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Characterization of *Bacillus cereus* BM1 with Protease Activity

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Microbial alkaline proteases dominate the world enzyme market, accounting for nearly two-thirds shares of the detergent industry. To date, Bacillus species have been known to produce a substantial amount of extracellular proteases which gain application in commercial industry. A protease producing bacterium was isolated from the eye of sea bass, for the first time, in an attempt to search for microbial biocatalyst that is detergent compatible. The isolate was characterized based on 16S rRNA gene sequence homology and Biolog Gen III microplate system. The results for identification indicate that the isolate has 97% sequence identity to *Bacillus cereus* with regard to 16S rRNA gene sequence homology which ultimately tally with the result of Biolog system, and hence designated as *Bacillus cereus* BM1. Moreover, protease produced by the isolate BM1 was assayed according to an established method. Subsequently the protease was partially characterized on the basis of temperature and pH requirements. Further characterization evaluated the effects of different metal ions (5mM), EDTA (5mM), NaCl (Up to 15%w/v) and commercial detergent (up to 10%w/v) on protease activity and/or stability. The results indicate that the *Bacillus cereus* BM1 produced a protease that is stable in alkaline pH range of 8-12, with optimum at pH 8 when incubated at 60°C for 1 hr. The protease was also stable at temperature ranges from 40-70°C, with optimum at 60°C when incubated for 1 hr. It shows activity in the presence of EDTA, as metal chelator suggesting that it is not a metalloprotease. Furthermore, none of the metal ions tested enhances protease activity above 100% from the control. The protease was also found to be stable in the presence of NaCl and commercial detergent. The results of partial characterization of the enzyme indicate that it is an alkaline, thermostable, halotolerant and detergent compatible non metalloprotease. This suggests that it will find application in detergent industry.

Key words: *Alkaline, Bacillus cereus, Biolog Gen III, Detergent, Protease and 16S rRNA.*

The plant and animal proteases are scarce in nature and therefore could not meet current world demands; this has led to an increased interest in microbial proteases. Bacteria and fungi are considered as ideal candidates that represent an outstanding source of enzymes because of their broad biochemical diversity and their simplicity to genetic manipulation. Approximately 40% of the total worldwide enzyme sales have been dominated by proteases from microbial origin (Godfrey *et al.,* 1996). Proteases from microbial sources are unique and becomes of special interest to the enzymes from plant and animal sources since they possess nearly all the characteristics required for their applications in biotechnology (Rao *et al.,* 1998).

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Bacterial proteases are considered to be of utmost significance. This is because neutral and alkaline proteases are of commercial significance, and the genus *Bacillus* is the most dominant producers. Alkaline proteases produced are of special interest as they could be used in manufacture of detergents, food, pharmaceuticals and leather (Saeki *et al*., 2007; Dias *et al*., 2008). Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10, and their broad substrate specificity. Their optimal temperature is around 60ºC. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry (Rao *et al*., 1998).

In recent years a number of studies have been conducted to characterize alkaline protease from different microorganisms. However, many of these proteases applied to industrial purposes face some limitations such as low stability towards high temperature, high NaCl concentration, commercial detergents stability, alkaline pH stability and production cost of the enzymes with regard to various carbon and nitrogen sources from growth medium. In addition, only those microorganisms that produces reasonable amount of extracellular proteases can be taken into account for commercial exploitation. The need for newer protease producing bacteria is of utmost significance to address the current plights of protease production rate, stability and catalytic efficiency that will be compatible with a designated industrial application. The search for newer biocatalyst that can address some of the aforementioned bottlenecks in commercial industries, particularly detergent industry necessitates these findings.

Characterization of bacteria with protease activity is a preliminary step for screening of protease producing isolates from different sources. Several bacterial proteases from cultural organisms have been characterized (Pushpam *et al.,* 2011). Alkaline proteases producing bacteria are widely distributed since bacteria are cosmopolitan in nature (Kumar *et al*., 2011). Most of the bacteria that are known to produce proteases have been isolated from soil. Little attention has been given to isolation and characterization of bacteria with protease activity from aquaculture. In this research we report for the first time a protease producing *Bacillus cereus* BM1 from the eye of sea bass.

Phenotypic and biochemical tests are usually used to identify bacterial isolate in environmental and clinical settings (Clarridge, 2004). However, there are limitations to these assays, thus 16S rRNA gene sequencing and standardized Biolog Gen III (microplate) system are often utilised to identify the isolate BM1. The protease producing isolate was identified as *Bacillus cereus*, and designated as *Bacillus cereus* BM1. The protease produced by the isolate was partially characterized to determine its application in commercial industries.

Materials and Methods

Cultivation and Isolation of Protease Producing Bacteria: The Sea bass were obtained from an open fish farm located in *Pusat Penyilidikan Ternakan Air Payau 81550 Gelang Patah* Johor Bahru, Malaysia. The fish were kept in ice and transported to the research laboratory within 1 hour. The bacterium was initially isolated and stored on a nutrient agar slant which was kept at 4°C. The bacterium was seeded on a freshly prepared nutrient agar and incubated at 37°C for 24 hour. Growth was observed after incubation, it was subsequently sub cultured until pure colonies were obtained.

Screening for Protease Enzyme

Casein Plate Assay Method: The purified bacterial isolate was seeded on 10% casein nutrient agar and incubated at 37°C for 24 h. Some of the bacterial colonies have shown clear zone of casein hydrolysis. The strain that indicated maximum relative zone diameter was designated as protease producer and hence selected for further experimental studies (Almas *et al*., 2009).

Protease Production: The culture medium used in this work for the protease production as described by Almas *et al.*,(2009) contained (w/v) 3% nutrient gelatin, 0.8% nutrient broth, 0.5% casein, 0.01% $MnCl_2$ and 1.2 ml of 20% glycerol, maintained at 37°C for 24 - 72 h. The above medium (100ml culture in 250ml Erlenmeyer flask) was maintained at 37°C for 24 - 72 hours in a shaking incubator (150 rpm). After 72 h of growth, the cells were harvested at 7043 × g for 15 min and the supernatant obtained was used as crude enzyme preparation. The samples were withdrawn at desired intervals, the optical density (OD) at A_{600nm} and enzyme activities at A_{440nm} were measured using 100 VIS spectrophotometer (Buck Scientific, USA).

Protease Assay: The proteolytic activity was determined by caseinolytic modified method as described previously by Kunitz, (1947) with azocasein as a substrate. Prepared crude enzyme (1ml) was mixed with 1% azocasein (Sigma-Aldrich) solution(1ml), that was dissolved in 0.02M Tris-HCl (trihydroxyaminomethane hydrochloride) buffer (pH 8.5) and was incubated in a water bath at 55°C for 10 min. The reaction was then terminated by the addition of 1 ml of 5% Trichloroacetic acid (TCA). The mixture was kept at 4°C for 15min. The preparation was centrifugation at 1127 × g for 20 minute and thus, supernatant (1ml) obtained was mixed in 0.4 M NaOH (1ml) and the absorbance was recorded at A_{440nm} using 100 VIS Spectrophotomer (Buck, scientific.USA). The blank contained TCA before incubation at 55°C. One unit of protease activity is defined as the amount of enzyme that produces an increase in absorbance of 0.01 under assay conditions.

Characterization of Protease Producing Isolate

Observation of Colony Morphology: The isolate was grown on three different media for preliminary morphological characterization. The media used were nutrient agar, thiosulphate citrate bile sucrose (TCBS), Eosin-methylene blue (EMB) and tryptic soy agar (TSA).

Determination of Temperature Growth Range: The temperature growth range of the isolate was investigated by inoculating the bacterium in nutrient broth for 24 - 48 hours at different incubation temperatures i.e 30, 37, 40, 70 and 80°C. The optical density (OD) at A_{600nm} of the isolate was measured using 100 VIS spectrophotometer (Buck scientific, USA) after 24 and 48 hours respectively.

Determination of pH Growth Range: The pH growth range of the isolate was investigated by growing the bacterium in nutrient broth for 24-48 hours at optimum temperature of 37°C. The medium pH adjustment was achieved by adding 1M NaOH and 1M HCl, using a digital pH meter before sterilizing the medium. The pH range of 1-10 was used during these studies. The optical density (OD) of the bacterium was measured at A_{600nm} using 100 VIS spectrophotometer (Buck scientific, USA)

Genomic DNA Extraction

The isolate was grown overnight in LB broth for 20h in a shaking incubation at 37°C prior to DNA extraction. The genomic DNA was isolated by using Promega kit. The protocol according to manufacturers was strictly adhered to. The DNA sample (1µl) was quantified using the nanodrop 1000 Spectrophotometer (Thermo scientific, USA). A total of 150 ng/µl of DNA was recorded, and then subsequently used for PCR reactions.

PCR Amplification

The 16S rRNA gene was amplified using Eubacterial universal primers: forward [5′AGAGTTTGATCCTGGCTCAG-3] and Reverse [5′GGTTACCTTGTTACGACTT-3′] as described by (Dubey et al., 2010). The PCR was conducted using PCR reaction mixture as follows: PCR master mix (promega), 24µl, DNA template (150ng/µl) 5µl, forward and reverse primers 5µl each

and sterile distilled water 11µl, which made up a total of 50µl. The cycling conditions were 1 cycle 95°C 4 minutes, 30 cycles of 95°C 5 sec, 55°C 45 sec and 75°C 78sec followed by final 5 minutes extension cycle at 75°C and hold at 4°C for 5 minutes. The PCR products were purified using QIAquick PCR purification kit (Qiagen, Hilden Germany), following strictly manufacturer's specification. The purified products were analysed using 1% agarose gel electrophoresis and visualised with UV irradiation after staining with ethidium bromide.

DNA Sequencing & BLAST

Both strands of the amplified PCR products containing the forward and reverse primers were sequenced by First BASE Laboratories Sdn Bhd (Shah Alam, Selangor, Malaysia). DNA sequences generated after sequencing were edited and assembled using Bioedit software package (Hall, 1999). After analyzing and assembling the respective sequences, a consensus sequence was used to query the NCBI BLAST database at NCBI to know the identity of the isolate under investigation.

Biolog Gen III Microplate™

The GEN III MicroPlate™ test panel was used which provides a standardized micro method using 94 biochemical tests to profile and identify a broad range of Gram-negative and Gram-positive bacteria. Biolog's Microbial Identification Systems software (e.g. OmniLog® Data Collection) was used to identify the bacterium from its phenotypic pattern in the GEN III MicroPlate.

Partial Characterization of Crude Protease Enzyme

Effect of Metal ions and EDTA on Protease Activity:

The effects of metals ions and EDTA were determined as described previously by Ghorbel *et al.,* (2003). The effects of metal ions (5mM) were investigated using the salts of Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+}. These salts are $CaCl_2$, $MgSO_4$, $MnCl_2$, $ZnSO_4$ and $CuSO_4$. The effect of protease inhibitor was studied using ethylene diamine tetraacetic acid (EDTA). Enzyme was pre-incubated with EDTA for 15 min at 20°C and then the residual activity was tested using azocasein (Sigma-Aldrich) as substrate.

Effect of pH on Protease Activity: The effect of pH on protease activity was determined as described by Ghorbel *et al.* (2003), briefly- the pH optimum of the crude preparation was studied over a range pH 6.0–12.0 with azocasein (Sigma-Aldrich) as substrate. The pH stability of the enzyme was determined by incubating the enzyme in buffers of different pH in the ranges of 6.0–12.0 for 1h, at 60°C. Aliquots were withdrawn and proteolytic activity was determined at pH 8.0 and 60°C. Buffer systems used in this study entails: Tris–HCl buffer for pH 7.0–9.0; 100mM potassium phosphate buffer for pH 5.0–8.0; and glycine-NaOH buffer for pH 9.0–12.0.

Effect of Temperature on Protease Stability: The temperature stability of the protease enzyme was investigated in accordance with the method described by Ghorbel *et al*., (2003), the effect of temperature on crude enzyme preparation was investigated for 30 miin at pH 8.0 Determination of thermal stability was conducted by incubating the enzyme for 30 min at different temperature. Thermal inactivation was examined by incubating the crude enzyme at 40, 50, 60 and 70°C for 30 minute. Samples were withdrawn to test the remaining activity at standard conditions. The enzyme incubated at 37°C was considered to be a control and was assumed to have 100% activity. The residual enzyme activity was determined using azocasein (Sigma-Aldrich) as substrate as described in the previous section.

Effect NaCl on Protease Stability: Sodium chloride (NaCl) effect on protease stability studies was conducted as described earlier (Kumar *et al.,* 2011). The effect of NaCl on

protease stability was assayed using NaCl solution of 1.0, 5.0, 10.0 and 15.0% (w/v) concentrations. Enzyme was diluted with equal volume of each solution and incubated for for 30 min at 60±1°C. Subsequently, the residual protease activity was assayed under standard conditions using azocasein as substrate.

Effect of Commercial Detergent on Protease Stability: Commercial detergent (Fabs Perfect) was employed to study its effect on protease stability according to the method of Kumar *et al*. (2011). Detergent solution was prepared at 0.1, 1.0, 5 and 10% (w/v) concentrations in distilled water. The crude enzyme was mixed with equal volume of each solution, incubated for 30 minutes at 60±1°C and assayed for residual protease activity under standard conditions.

Results & Discussion

Screening and Isolation of Bacteria:

Seven isolates were screened and only three of them showed protease activity. Among the three isolates, an isolate denoted "BM1" showed highest activity was selected and further used for the study. The pure colonies of the isolate BM1 were obtained by repeated streaking using agar plate technique

Pure colonies of the bacterium were obtained by repeated streaking on nutrient agar. The preliminary characterization of the bacterium was performed by screening for caseinolytic activity. The bacterium from the eye of sea bass showed highest and/or unique proteolytic activity among others. This is evident by its ability to produced casein hydrolysis on casein agar plate. On the basis of its unique protease activity the isolate BM1 was selected for further experimental studies. The evidence of casein hydrolysis has been dominant among genus *Bacillus,* and this finding is in agreement with the work of Almas *et al.* (2009), who reported a protease producing *Bacillus* sp on casein agar plate by hydrolysis.

Protease Production

Figure 1 shows the time-course of protease production by *Bacillus cereus* BM1 in liquid medium containing 3% nutrient gelatine, 0.8% nutrient broth and 0.5% casein maintained at 37°C for 26 hours in 250ml Erlenmeyer flask at 150 rpm in shaking incubator. A formation of protease significantly started from early stationary phase and reached a maximum in 24 hours and then began to fall.

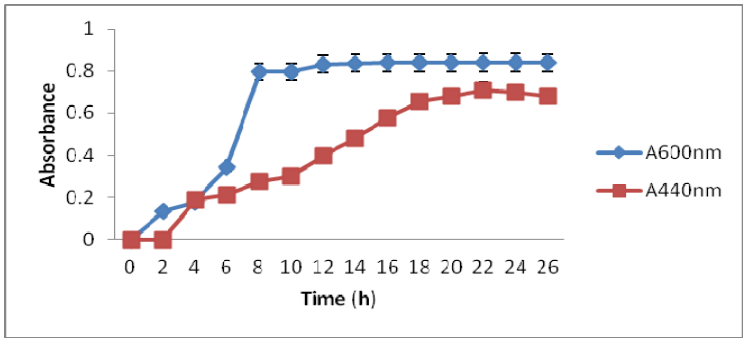

Figure 1: Protease production as a function of cultivation time by *Bacillus cereus* BM1. Results represent the means of three experiments. The bar indicates variation from the mean by not more than 5%

The time-course of protease production by *Bacillus cereus* BM1 in liquid medium as described previously (Almas *et al*., 2009) in 250mL Erlenmeyer flasks has been depicted in Figure 1. The formation of protease significantly started from early

stationary phase and reached a maximum in 24h, and then began to fall. This result is in conformity with work of Sepahy *et al.* (2011) who reported the maximum protease activity at 24 hours of *Bacillus* sp. Strain CR-179. In a similar study maximum protease activity was determined at the 18th hour, which occured in the late stationary phase, when most of the bacteria sporulated (Özgür, K. and Nilüfer C, 2011). *Bacillus* sp. is a spore forming bacterium, thus during sporulation and also germination, it increases protease activity (Sepahy *et al.*, 2011). Scientists acclaimed that during sporulation and germination, hydrolyzed proteins were used to compose proteins for endospores or vegetative cells (Prestidge *et al.*, 1971; James *et al.*, 1985). This process requires an increase of protease production. This is in contrast to previous report which showed that *Bacillus* sp usually produce more protease during the late exponential phase (Sepahy *et al.*, 2011). Another investigation done by Asokan and Jayanthi (2010) revealed different results; they observed that the optimum incubation time for enzyme production is 96 hours

The present findings pave way for the optimization and/or search of cost effective growth medium for the production of alkaline proteases from *Bacillus cereus* BM1. This is because; there is a need to find new strains of bacteria with the ability of producing proteolytic enzymes with novel properties and the development of low cost media.

pH and Temperature Growth Profile of the Isolate BM1

The culture density of the isolate BM1 at various pH and temperatures were investigated. The results showed optimum pH at 7 but reasonable culture density were also recorded at broad pH ranges of 5-9 (Figure 2). The results for the temperature requirement of the isolate BM1, on the other hand revealed optimum activity at 40ºC but culture density was recorded at 30, 37, 70 and 80ºC.

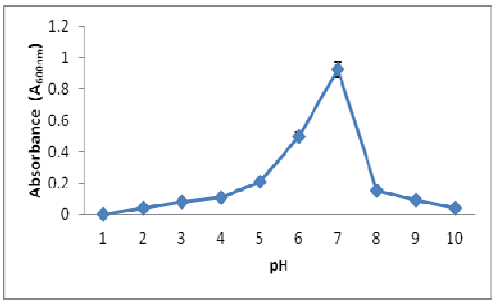

Figure 2: Effect of pH on *Bacillus cereus* BM1 growth at A_{600nm}. Results represent the means of three experiments. The bar indicates variation from the mean by not more than 5%.

The pH and temperature growth ranges of the isolate were determined using established methods. The result of *Bacillus cereus* BM1 in relation to pH showed broad range of pH activity with optimum at 7 (Figure 2). The pH requirement of the isolate is extremely important as it is considered as one among the factors that inflences the growth and enzyme production. The best optical densisty was recorded at pH 7. On the other hand, the temperature growth range of the isolate BM1 was found to be optimum at 40°C, but activity were recorded at 30, 37, 70 and 80°C (Figure 3).

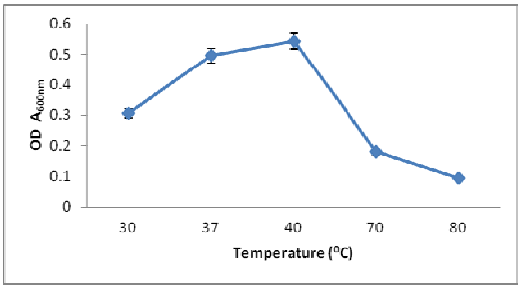

Figure 3: Effect of temperature on *Bacillus cereus* BM1 growth at A_{600nm}. Results represent the means of three experiments. The bar indicates variation from the mean by not more than 5%.

16S rRNA Gene Sequence Homology:

The primers are highly conserved among prokaryotes and are designated as eubacterial universal primers as described by (Dubey *et al.*, 2010). In addition they

were found to amplify the whole region of the rRNA gene, which is 1500 bp (Figure 6). The PCR product sequencing was done by First BASE Laboratories Sdn Bhd (Shah Alam, Selangor, Malaysia). The DNA homology search on the GenBank database (http://www.ncbi.nih.gov) was performed. Phylogenetic and molecular evolutionary analyses were conducted using Mega 4.0 (Tamura *et al.*, 2007).

A phylogenetic tree was constructed based on the comparison of the 16S rDNA sequence of this isolate and other strain of *Bacillus*. All the sequences were aligned with CLUSTALW from MEGA 4 software package. Other 16S rDNA sequences of *Bacillus* were obtained from the GenBank database (http://www.ncbi.nih.gov.). The BLAST results (not shown) gathered from the 16S rRNA analyses show that *Bacillus cereus* BM1 is very close to *Bacillus cereus strain* JY2 and *Bacillus cereus* strain JY7, (Figure 4). The partial sequencing of the 16S rRNA shows a 97% similarity to different strains of *Bacillus cereus* (Figure 4).The 16S rRNA sequence of *Bacillus cereus* BM1 was deposited in NCBI and was assigned Genbank accession number of KC736480.

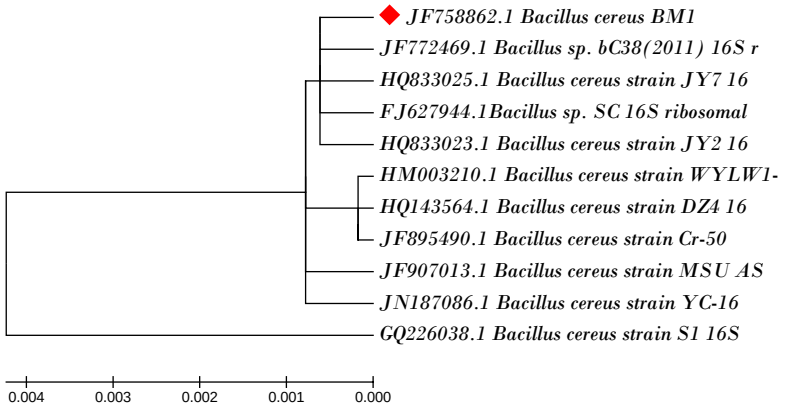

Figure 7. Phylogenetic position of *Bacillus cereus* BM1 with other Bacteria. The members of bacteria used include *Bacillus sp* bC 38; *Bacillus cereus* strain JY7; *Bacillus sp*. SC; *Bacillus cereus* strain JY2: *Bacillus cereus* WYLW1; *Bacillus cereus strain* DZ4; *Bacillus cereus* strain Cr-50; *Bacillus cereus* strain MSU; *Bacillus cereus* strain YC; *Bacillus cereus* S1. Phylogenetic tree was inferred by using the neighbour-joining methods. The software package MEGA4 was used for the analysis. The phylogenetic analysis revealed that the closest relatives of the isolated strain were *Bacillus cereus.*

Biolog Gen III Microplate™ and Characteristics of the Isolate BM1

The Biolog GEN III 2_6_1_08.15G (Focus Biotech, Selangor, Malaysia) was used to analysed the isolate BM1 on the basis of 94 phenotypic tests which entails different biochemical profiling such as carbon source utilization assays (71) and chemical sensitivity assays (23) to confirm the strain identity of *Bacillus cereus* BM1 as shown by 16S rRNA BLAST results. After incubation, as described previously, the phenotypic finger print of the positive wells was compared to Biolog's extensive species library, which tally with *Bacillus cereus*. This result is in agreement with the 16S rRNA sequence homology (97% to *Bacillus cereus*). The result of these analyses indicates that the isolate was positive to D-Glucose, D-fructose, glycerol, gelatine, D-serine and L-serine etc, (Table 1). The positivity of the isolate BM1 to the serine(s) is an indication that the protease enzymes secreted by this isolate may likely be a serine protease. In addition, the strongly reactive carbon sources results (Table1) can be considered for effective optimization for protease production in future work.

Further more the chemical sensitivity assays indicates that the isolate

BM1 showed salt tolerance and metabolic activity at 1, 4 and 8% NaCl concentrations. On the basis of this finding, *Bacillus cereus* BM1 will be described as halotolerant bacterium. It also showed positive activity at pH 6 but no activity at pH 5. The pH result of the GENIII microplate system tally with the one determined using the conventional system. This is evident by its ability to grow in nutrient broth with pH 6 and optimum activity at pH 7 (Figure 2).

Strain BM1 is an aerobic, rod-shaped, with irregular colony shape and gram positive bacterium. It was found to be catalase positive with ability to hydrolyse casein on 10% casein agar medium. Strain BM1 has the ability to grow on different media such as EMB, TCA, but showed no growth on TCBS. The preliminary characterizations of the strain BM1 are listed in Table 1.

Table 1: Preliminary Characterization of *Bacillus cereus* BM1

Characteristics	Observation
Colony colour	White, it gradually changes to red as the cultures grow old.
Colony Margin	Irregular
Colony Elevation	Convex
Gram Staining Reaction	Positive
Cellular Shape	Rod or Bacilli
Cultivation Media	NB, LB, TSA & NA. No growth on TCBS & EMB
Optimum growth Temperature	40 ± 2°C
Optimum growth pH	7.0
Casein Hydrolysis	positive
Gelatine Hydrolysis	Positive

Two methods, including automatic bacterial identification system (Gen III microplate) and 16S rRNA gene homology were used to identify the isolate BM1 because some bacteria have ambiguous biochemical profile. However, 16S rRNA gene sequences of some bacteria are still limited which will affect the accuracy of the identification results. For all the reasons above, in order to obtain objective and reliable identification results different approaches and/or methods should be used to mutually support bacterial identification results.

According to the 16S rRNA analysis, the Biolog system results and preliminary morphological properties of the bacterium, it was identified as *Bacillus cereus* and hence designated as *Bacillus cereus* BM1.

Partial Characterization of *Bacillus cereus* BM1 Protease

Effect of Metal Ions and EDTA on Protease Activity:

The effect of different metal ions (5 mM) on protease activity at 60°C was studied. Mg^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+} and Ca^{2+} slightly decreased the protease activity by 85, 80, 91, 82, 71% of the control, respectively (Table 2). Ghorbel *et al.* (2003) reported a similar finding but with Ca^{2+} as the best inducer, while Zn^{2+} and Cu^{2+} as inhibitors which is contrary with this findings. Surprisingly, in the presence of metal chelator (EDTA), the enzyme retained its activity up to 97%, suggesting that it is not a metalloprotease. This might be the possible reason why all the metal ions tested in this study does not increase the activity of the enzyme, but rather greater percentage of the protease activity was retained in the presence of these metal ions, as such the enzyme may likely require no metal inducer or cofactor for optimum activity. Doddapaneni *et al.* (2009), also reported a *B. cereus* protease that was inhibited by EDTA and Cu^{2+} enhanced the activity of the protease by four fold, indicating that it is a metalloprotease. This is contrary to this study.

Rao *et al.* (1998), reported that subtilisin (Carlsberg) enzyme has a broader substrate specificity and does not depends

on Ca^{2+} for its stability, this is in agreement with this finding because no metal inducer is required for the stability of the protease under investigation. This may likely reduce the cost of enzyme production in case of industrial production.

Table 2: Effect of Metal ions & EDTA on Protease Activity

Metal ions /EDTA	Concentration (5mM)	Relative Activity (%)
None	-	100
Ca^{2+}	5	71
Mg^{2+}	5	85
Zn^{2+}	5	82
Cu^{2+}	5	80
Mn^{2+}	5	91
EDTA	5	97

Table 2 depicts the effects of metal ions and EDTA on protease activity. The enzyme preparation without any additive was considered as control. This was investigated to evaluate the stimulatory or inhibitory effects of these additives on protease activity. None of them showed stimulatory effect above the control but only slight inhibitory effect was observed among few additives (Table 2)

Effect of Temperature on Protease Stability:

Another remarkable and outstanding feature of the protease produced by *Bacillus cereus* BM1 is its stability at high temperatures. The protease appeared to be stable and was found to be able to retain its full activity after 30 min of incubation in the temperature ranging from 40 to 70°C (Figure 5). The crude enzyme showed stability at 40, 50, 60 and 70°C with the relative activity of 105.6, 103.6, 104.2 and 97.5% respectively from the control in the absence metal ions. This is suggesting that no metal ion inducer is required for optimum activity of the designated protease. Several workers (Ghorbel *et al.*, 2003 and Rahman *et al.*, 2009) reported the thermostability of their proteases with Ca^{2+} as inducer and the activity of their proteases drastically reduced at 70°C. To our knowledge, this is the first protease from *Bacillus cereus* BM1, with relative activity of 97.5% from the control at 70°C without an inducer. Furthermore, Doddapaneni *et al*. (2009), reported a protease from *B. cereus* with optimum activity at 60°C, which is completely in agreement with the optimum temperature (60°C) for the protease under investigation in this study. On the basis of this finding, the *Bacillus cereus* BM1 protease can be considered as moderately thermo active protease, thus it can be suitable to be used in industrial and biotechnological applications.

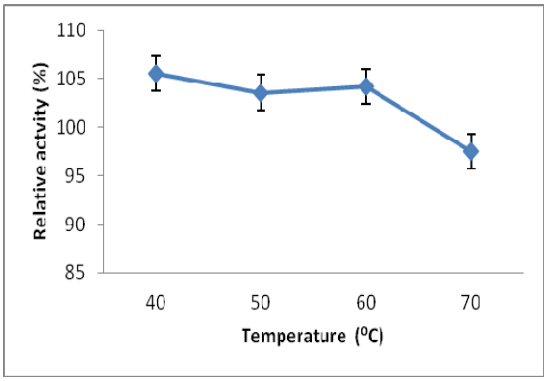

Figure 5: Effect of Temperature on Protease Stability. The crude enzyme preparation was incubated at various temperatures (37-70°C) for 30 minutes. Protease activity at 37°C was considered as 100%. Results represent the means of three experiments. The bar indicates variation from the mean by not more than 5%.

Effect of pH on Protease Activity:

The relative activity values at various pHs are shown in Fig.6. The optimum pH for protease activity determined at 60°C was 8.0. The enzyme at pH 9, 10, 11, and 12 was stable with relative activity of 104.2, 90.65, 103.35 and 99.82% respectively from the control.

The pH stability was determined by incubating the enzyme in buffers of different pH in the range of 5.0–12.0 for 1 h at 60°C, followed by activity estimation at pH 8.0. As shown in Fig. 4.10, the enzyme was stable between pH 5.0 and 12.0, and

retained more than 100% of its original activity, after incubation at 60°C for 1 h at pH 8, 9 and 11 in the absence of any metal inducer or cofactor. Ghorbel *et al.* (2003) reported proteases from *Bacillus cereus* BG1 with optimum pH at 8 in the presence of Ca^{2+} and its activity drastically declined at pH 10, 11 and 12, which slightly tally with optimum pH of the protease under investigation. In the same vein, Doddapaneni *et al.* (2009) reported a protease from *B. cereus* with optimum activity at 10, but its activity declined at pH 11, 12 and 13. On the contrary, the protease from *Bacillus cereus* BM1 was viable within a broad pH ranges from 5.0-12.0, with optimum at 8 (Figure 6). This finding is directly indicating that the enzyme can be designated as an alkaline protease.

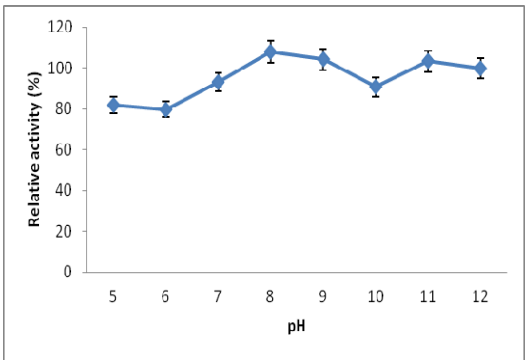

Figure 6: Effect of pH on protease activity. The crude enzyme preparation was incubated at different pH (5 – 12) for an hour at 60°C. Protease actvity in the absence of any buffer is considered as 100%. Results represent the means of three determination/experiments. The bar indicate variation from the mean by not more than 5%. Absence of bars indicates that errors were smaller than symbols.

Effect of NaCl on Protease Stability:

Bacillus cereus BM1 produces a protease with almost full stability and activity at 1%, 5%, 10% and 15% (w/v) NaCl concentrations, with relative activity of 84.35, 78.83, 149.63 and 94.17% respectively from the control (Fig 7). Surprisingly, an optimum activity was observed at 10% salt concentration with relative activity of 149.63% from the control. Kumar *et al.* (2011) reported a protease which an increase in salt concentration resulted in gradual decrease in activity, and retained 92, 84, 68, 52 and 48% activity at 4, 5, 7, 9 and 10% salt level, respectively. Higher than 10% NaCl resulted in drastic decrease with residual activities of 18 and 6% at 11 and 12% (w/v) salt concentrations, respectively. The effect of common salt on BM1 protease revealed highly halo-tolerant nature of the enzyme, which was independent of NaCl requirement. This finding is interesting as the isolate is from non saline environment. Joshi *et al*. (2007) have reported a *B. cereus* isolate from a lake having protease active up to 5% NaCl concentration, but lost its activity with increasing salinity. Joo and Chang (2005) have also reported an alkaline protease from a halo-tolerant *B. clausii* I-52, active in a broad range of NaCl concentration, but most active only at 1% NaCl. To our knowledge, we report for the first time a halo-tolerant protease from *Bacillus cereus* BM1 with over 100% activity from the control at 10% salt concentration. The stability in the presence of high salt concentration is a very important characteristic as NaCl is used as core component in granulation of protease prior to addition in detergents. The quality of ground water available in some parts of the world is saline (Kumar *et al*., 2011). The salinity is detrimental or harmful for washing properties of the detergent; hence, the presence of halo-tolerant alkaline protease becomes extremely important characteristics with respect to application in detergent industries.

Effect of Commercial Detergents on Protease Stability:

The protease was suitably stable to the extent of 98.2 % in the presence of 0.1% (w/v) commercial detergents. Upon 10 fold increase (at 1%) in commercial detergents' (Fabs perfect) concentration, the activity of protease increased to 116%. Furthermore, 50 fold increases (at 5%) in commercial

detergents' concentration, the protease showed remarkable increase in residual activity of 298.9% thereby indicating its high performance and stability in commercial detergent (Figure 8). Even at 10% (100 fold increase) detergent level, the residual protease activity was 178% in Fabs perfect.

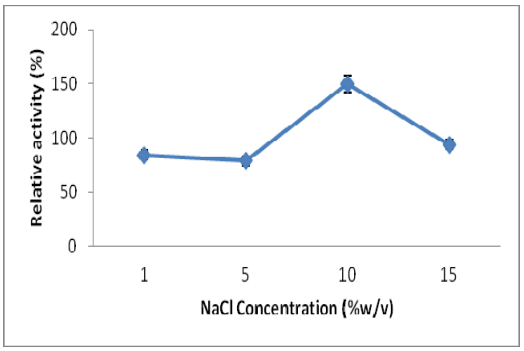

Figure 10: Effect of NaCl on protease Stability. The crude enzyme preparation was incubated at different NaCl concentrations for 30 minutes at 60±1°C. Protease activity in the absence of any additive was considered as 100%. Results represent the means of three experiments and the bar indicate variation from the mean by not more than 5%. Absence of bars indicates that errors were smaller than symbols.

Pattern of protease stability in the presence of commercial detergents (Figure 8) also reveals its possible commercial application in detergent formulations. Generally, most of the manufacturers recommend the use of detergents at 0.1 to 0.2% (w/v) ranges for washing purposes. Accordingly, the halotolerant thermo-alkaline protease from *B. cereus* BM1 is highly stable and showed considerable increase in the afore mentioned recommended ranges. Detergent stable proteases have been studied by several groups of workers with varying levels of activity in the presence of different detergents (Joo and Chang, 2005; Kuddus and Ramteke, 2009). Detergent stability of an alkaline protease is an important property for its industrial use, as they are currently supplemented in detergent formulations for better washing efficiency.

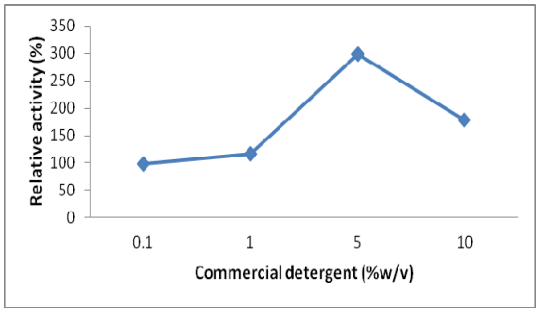

Figure 8: Effect of commercial detergent (Fabs perfect) on protease stability. The crude enzyme was incubated at different detergent concentrations for 30 minutes at 60±1°C. Protease activity in the absence of commercial detergent was considered as 100%. Results represent the means of three experiments and the bar indicate variation from the mean by not more than 5%. Absence of bars indicates that errors were smaller than symbols.

Conclusions

Alkaline proteases constitute very huge and complex groups of enzymes, with both regulatory and nutritional roles in nature (Gupta *et al.*, 2002b). They are produced by myriad microorganisms but most exploited industrial producers belong to the genus *Bacillus* (Gupta *et al.*, 2002a). A protease producing *Bacillus cereus* BM1 has been isolated and characterized for the first time from the eye of sea bass. The isolate was characterized on the basis of 16S rRNA gene sequence homology, pH and temperature requirements, carbon source utilization and chemical sensitivity assays (Biolog system, Focus Biotech, Malaysia). In addition, it was ascertained that substantial amount of extracellular protease can be produced by the isolate BM1 within first 24 hours of incubation. Given its potential industrial applications, protease produced in this study has been partially characterized to evaluate its thermo-stability, and detergent compatibility with respect to halotolerant, alkalophilic nature of the enzyme. The BM1 protease can be conclusively regarded as thermostable,

alkaline, halotolerant and non metallo-protease with no metal ion requirement for maximum activity. The fact that the enzyme is stable in reasonable NaCl and commercial detergent concentrations made it feasible to be use as detergent formulation to improve washing efficiency.

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