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Production, Partial Purification, Characterization and Detergent Compatibility of Alkaline Protease from Soil Isolate *Bacillus cereus* AG1

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A B S T R A C T

Proteases are one of the most important groups of enzymes with wide range of industrial applications. The soil isolate *Bacillus cereus*AG1 produced alkaline protease under submerged cultivation condition. Maximum production of enzyme was achieved at pH 10.0 and temperature 30^{o}C at 120 rpm. The supplementation of fructose as carbon source and combination of beef extract, yeast extract and peptone as nitrogen source improved enzyme production by 1.39 times. Partial purification of enzyme by dialysis exhibited 3.07 fold purification with 60.15% yield. The characterization of partially purified alkaline protease displayed optimum activity at pH 10.0, temperature 50^{o}C with casein as the most suitable substrate. Mmaximum enzyme activity 250 U ml^{-1}and Michaelis constant (0.28%) of alkaline protease were obtained by varying casein concentration (1.0-2.0% w/v). The alkaline protease activity was enhanced in the presence of Ca^{+2}, while Na^{+} and Hg^{+2} inhibited the enzyme activity. The enzyme retained 59.92% activity in the presence of 5.0 mM EDTA. The detergent stability and compatibility of alkaline protease suggest its potential utility as cleaning additive.

Introduction

Proteases represent large and complex group of enzymes found in all living organisms and play an important role in normal and abnormal physiological conditions (Burhan *et al.*, 2003, Asha and Palaniswamy, 2018). Serine protease (EC. 3.4.21), cysteine (thiol) protease (EC.3.4.22), aspartic protease (EC. 3.4.23) and metallo-protease (EC.3.4.24) are among the most valuable commercial enzymes contributing approximately 60% of the total industrial market worldwide (Nascimento and Martins, 2004, Verma *et al.*, 2011). Owing to vast diversity in physico-chemical and catalytic properties, alkaline proteases show increased applications in detergent, food processing, pharmaceutical, peptide synthesis, meat tenderization, medical diagnosis, baking and brewing industries (Genari *et al.*, 1998,

Bayoudh *et al.*, 2000). The inherent properties like stability and activity in alkaline condition makes alkaline protease first choice in the preparation of enzyme based cleaning formulations as well as laundry detergent additives (Venil and Lakshmanaperumalsamy, 2009). The modern enzyme detergents often referred to as "Green Chemicals" are efficient, environmental friendly and economical strategy useful in degradation of unwanted protein stains along with keeping a check on the environmental pollution (Vishalakshi *et al.*, 2009). The detergent compatibility and applicability of alkaline protease for the cleaning of blood, food and egg stains is well documented (Banerjee *et al.*, 1999, Adinarayana *et al.*, 2003, Wang *et al.*, 2007, Kalpana Devi *et al.*, 2008).

Among several source organisms, *Bacillus* spp. are the most explored for the commercial level production of alkaline proteases. The alkaline proteases derived from *Bacillus* sp. are highly active and stable at robust conditions of pH and temperature. They show broad substrate specificity and low purification cost (Haddar *et al.*, 2009). Extracellular production of alkaline protease by microorganisms is greatly influenced by several cultivation parameters like pH, temperature, aeration, inoculum density, incubation time, carbon, nitrogen source, salt and metal ion concentration (Gupta *et al.*, 2002; Jasvir *et al.*, 2004). The present study deals with isolation and optimization of culture conditions for hyper production of alkaline protease from *Bacillus cereus* AG1. Further, partial purification characterization and detergent compatibility was carried out.

Materials and Methods

Chemicals and reagents

All chemicals and reagents used, unless stated otherwise, were of analytical grade and procured from Sisco Research Laboratory (SRL) Pvt. Ltd. India, Central Drug House (CDH) Chemicals Pvt. Ltd. India and Himedia Pvt. Ltd., India.

Nutrient media

Nutrient agar (NA) containing g l^{-1}: peptone, 10.0., beef extract, 3.0., NaCl, 5.0., agar-agar, 30.0., and pH 7.0 ± 0.2 was used for isolation and maintenance of bacterial cultures. The modified basal medium (MBM) containing g l^{-1}: glucose, 10.0., peptone, 2.5., beef extract, 1.5., yeast extract, 2.5., NaCl, 5.0., and pH 10.0 ± 0.2 was used for submerged production of alkaline protease.

Isolation and screening of protease producing microorganisms

The isolation of alkaline protease producing bacterial cultures was performed from soil of different locations of Anand such as gardens, compost sites, agriculture fields, petrol pumps and wastewater treatment plant. The soil samples were collected from 6.0 cm depth using sterile spatula and transferred to sterile polybag. The collected soil samples were serially diluted in sterile distilled water, plated on nutrient agar plates and incubated at 30^{o}C for 24-48 h. The well isolated bacterial colonies with distinct characteristics were further transferred on nutrient agar slants and maintained at 4^{o}C.For screening of alkaline protease production, all the bacterial cultures were streaked on casein agar plates (pH 10.0) and incubated at 30^{o}C for 24 h. The plates were flooded with solution containing l^{-1}: $HgCl_2$, 150.0, HCl, 200.0 ml and alkaline protease production was determined by formation of clear zone around colonies.

Alkaline protease production in liquid medium

The production of alkaline protease was carried out in 250 ml Erlenmeyer flasks containing 100 ml MBM. The media were

inoculated with 3% (v/v) pre grown active bacterial cultures with uniform cell density (OD_{600} 1.0). All the flasks were incubated at 30^{o}C for 48 h in shaking condition (120 rpm). The aliquots (2.0 ml) were withdrawn from different time intervals, centrifuged at 10,000 rpm for 10 min and clear supernatant was processed for enzyme assay.

Identification of bacterial cultures

Primary identification of potential bacterial cultures was done by microscopic findings (gram staining, endospore staining) and growth characteristics. Further, various physiological and biochemical properties of selected culture were investigated according to Bergey's Manuals of Systematic Bacteriology. The final characterization of culture was done by 16S rDNA sequencing from Bangalore Genei Pvt. Ltd., Banglore, India.

Optimization of physico-chemical parameters for alkaline protease production

In order to evaluate the effect of various physico-chemical parameters on alkaline protease production, single factor at a time methodology i.e., varying a single factor at a time and keeping others constant was applied. The alkaline protease production was performed varying pH values (5.0-12.0) and temperature (15-50^{o}C). In the next step, the optimization of various carbon sources (1.0% w/v) and nitrogen sources (0.1% w/v) was checked for effective enzyme production by bacterial culture.

Partial purification and characterization of alkaline protease

The culture medium after incubation was centrifuged at 10,000 rpm for 15 min at 4°C to remove cells debris. The clear supernatant was placed in ice bath, mixed with solid ammonium sulfate (50-100 % saturation) as per standard chart and kept for overnight at 4°C.The resulting precipitates were collected by centrifugation at10,000 rpm for 20 min at 4°C. The pellets were dissolved in minimal volume of 50mM glycine-NaOH buffer (pH-10.0) and dialyzed against distilled water for several times to remove ammonium sulfate. The dialyzed enzyme concentrate was used for characterization of pH (5.0-12.0), temperature (30-80^{o}C), substrate (casein, gelatine, bovine serum albumin (BSA)), metal ions (Na^{+}, Ca^{+2}, Hg^{+2}) and inhibitors (β-mercaptoethanol, EDTA).

Alkaline protease assay

The proteolytic activity was determined spectrophotometrically according to described by Gessesse *et al.,* (2003). The reaction mixture containing 1.0 ml casein (1.0% w/v) in 50 mM glycine-NaOH buffer (pH-10.0) and 1.0 ml of suitably diluted culture supernatant was incubated at 50^{o}C for 20 min. The reaction was terminated by addition of the 2.0 ml trichloroacetic acid (10% w/v) and incubated at room temperature for 10 min. The content was centrifuged at 10,000 rpm for 10 min to remove precipitates of unreacted casein. The supernatant (1.0 ml) was used for protein determination by standard method of Folin-Lowry using tyrosine as standard (Lowry *et al.,* 1951). One unit of enzyme was defined as the amount of enzyme required to liberate 1.0 µg of tyrosine per minute under standard experimental conditions.

Detergent stability

The stability and compatibility alkaline protease with detergents was evaluated in the presence of $CaCl_2$ (10.0 mM) and glycine (1.0 M) (Adinarayana, 2003). Different detergents used were Nirma (Nirma chemical, India); Ariel and Tide (Procter and Gamble, India); Wheel (Hindustan Liver Ltd., India) and local

made detergent (Anand).The detergent solutions (1.0% w/v in distilled water) were incubated at 65^{o}C to inactivate endogenous proteases. The partially purified alkaline protease was mixed with detergent solution (1:4 ratio) and incubated at 50^{o}C for 3 h. After incubation, residual activity was determined under standard condition. The enzyme activity of control sample (without detergent) was taken as 100%.

Data analysis

All the experiments and the analysis were performed in triplicate and the data presented is the mean value of the triplicates. The standard error of mean (SEM) was calculated using the mean values and remained within the rangeof±10%.

Results and Discussion

Isolation, screening and identification of alkaline protease producing bacteria

The serial dilution and standard plate count method resulted into isolation of total 56 bacterial cultures. Upon primary screening on casein agar plate (pH 10.0), 12 cultures exhibited positive alkaline protease production in terms of clear zone around the colonies.

On the basis of ratio of diameter of zone of clearance (mm) and colony size (mm), isolate YPAG-18 displayed highest enzyme production (3.12±0.06) (Fig. 1A) followed by isolate YPAG-34(2.91±0.07) and YPAG-41 (2.56±0.04). Other isolates showed lesser degree of alkaline protease production. Hence, the isolate YPAG-18 was selected for further screening and identification. Morphological and cultural characteristics revealed that the isolate YPAG-18 was gram positive, endospore forming rods. The growth of isolate YPAG-18 on nutrient agar was as large, irregular, flat, rough, cream and opaque colonies (Fig. 1B). The isolate was further identified based on the methods described in Bergey's Manual of Systematic Bacteriology and finally identified as *Bacillus cereus* AG1 on the basis of 16S rDNA gene sequencing (Gene Bank accession no.GU433107) and deducing phylogenic identity (Fig. 1C).

The time course study of alkaline protease production by *Bacillus cereus* AG1 was performed under submerged condition at 120 rpm. Figure 2 demonstrated that the enzyme production increased in relation to incubation time and reached at maximum level (177±2.3 U ml^{-1}) after 24 h. Further incubation led to lower level of enzyme production. The bacterial growth was also highest (1.76 O.D.) at 24 h suggesting biomass dependent alkaline protease production. Many researchers described the production of alkaline protease by different species of *Bacillus* (Sharma *et al.*, 2014, Asha and Palaniswami, 2018).

Optimization of physico-chemical parameters for alkaline protease production by *Bacillus cereus* AG1

Effect of pH

In order to evaluate the effect of pH, the alkaline protease production by *Bacillus cereus* AG1 was performed in the range of pH 5.0–12.0. The maximum enzyme production was observed at pH 10.0(178±1.9 U ml^{-1}) and at pH 11.0 the enzyme production was 121±2.2 U ml^{-1}, which is also quite noteworthy (Fig. 3). Nevertheless, the alkaline protease production was significantly reduced at pH 5.0 (12±0.9 U ml^{-1}) and pH 12.0 (65±1.2 U ml^{-1}). At pH 6.0, 7.0, 8.0 and 9.0, the enzyme production was 46±0.8 U ml^{-1}, 88±1.4 U ml^{-1}, 103±2.1 U ml^{-1}and 155±1.9 U ml^{-1} respectively. The major influence of pH may be attributed to the enzymatic processes and nutrient transport across the cell membrane. In accordance to our results, Pant *et al.*, 2015

reported optimum pH 10.0 for alkaline protease production by *Bacillus subtilis*.

Effect of temperature

The effect of temperature on alkaline protease production was tested in the range of 15-50^{o}C.In our study, although *Bacillus cereus* AG1 was able to grow well at entire temperature range tested, it preferred30^{o}C temperature for maximum enzyme production (180±2.9 U ml^{-1}) (Fig. 4). The enzyme production was only 13.33% and 18.33% at the temperature 15^{o}C and 50^{o}C respectively. The enzyme production was 104±1.9 U ml^{-1}, 123±2.2 U ml^{-1} and 65±2.1 U ml^{-1} at 20^{o}C, 37^{o}C and 45^{o}C respectively. Above results suggested that temperature has profound influence on the production of alkaline protease by *Bacillus cereus* AG1. Although the mechanism of temperature control over alkaline protease production is poorly known, it influences the synthesis and secretion of enzyme by regulating energy metabolism and oxygen uptake, by translational synthesis of protein and by altering physical properties of cell membrane of microorganisms (Frankena *et al.,* 1986, Votruba *et al.,* 1991, Rahman *et al.,* 2005). Rathod and Pathak, 2016 reported maximum alkaline protease production at 30^{o}C by *Halomonasvenusta*LAP515.

Effect of carbon source

The effect of substitution of glucose in culture medium by different carbon sources on alkaline protease production by *Bacillus cereus* AG1is depicted in table 1. It was evident that fructose proved to be the most suitable carbon source with highest enzyme activity. The enzyme production was 1.25 times higher compared to commonly used carbon source glucose. The presence of maltose and sucrose displayed 76±1.6 U ml^{-1} and 70±2.1 U ml^{-1} enzyme activities respectively. On the other hand, the alkaline protease production was considerably reduced in the presence of sodium acetate. Our results are in good accordance with Mabrouk *et al.,* 1999, wherein maximum enzyme production by *Bacillus licheniformis* ATCC 21415 was achieved in the presence of lactose or fructose. Nevertheless, Gessesse *et al.,* 2003 reported increased level of protease production by *Bacillus pseudofirmus* AL-89 upon addition of glucose, whereas suppression of enzyme production by *Nesterenkonia* sp. AL-20 was obtained in the presence of glucose.

Effect of nitrogen source

An assessment of various organic and inorganic nitrogen sources on the production of alkaline protease by *Bacillus cereus* AG1 was carried out in the submerged condition. The results in table 2 suggested that incorporation of single nitrogen source in the medium did not influence alkaline protease production by *Bacillus cereus* AG1. The combination of beef extract, yeast extract and peptone on the other hand exhibited enhanced enzyme production, which may be owing to synergistic effect of these three organic nitrogen sources. It was evident that organic nitrogen source exhibited relatively superior effect on enzyme production as compared to inorganic nitrogen source. The alkaline protease production was rather inhibited in the presence of inorganic nitrogen sources. Different organic nitrogen sources like peptone, beef extract and yeast extract for the production of alkaline protease by *Bacillus* sp. have been documented by Ghafoor and Hasnain, 2009 and Marathe *et al.,* 2018.

Partial purification of alkaline protease

Ammonium sulphate precipitation of alkaline protease from *Bacillus cereus* AG1displayed highest enzyme activity as well as specific activity at 80% saturation. The obtained purification fold was 1.49.

Fig.1 Zone of proteolysis (A), growth characteristics (B) and phylogenic relationship (C) of *Bacillus cereus* AG1

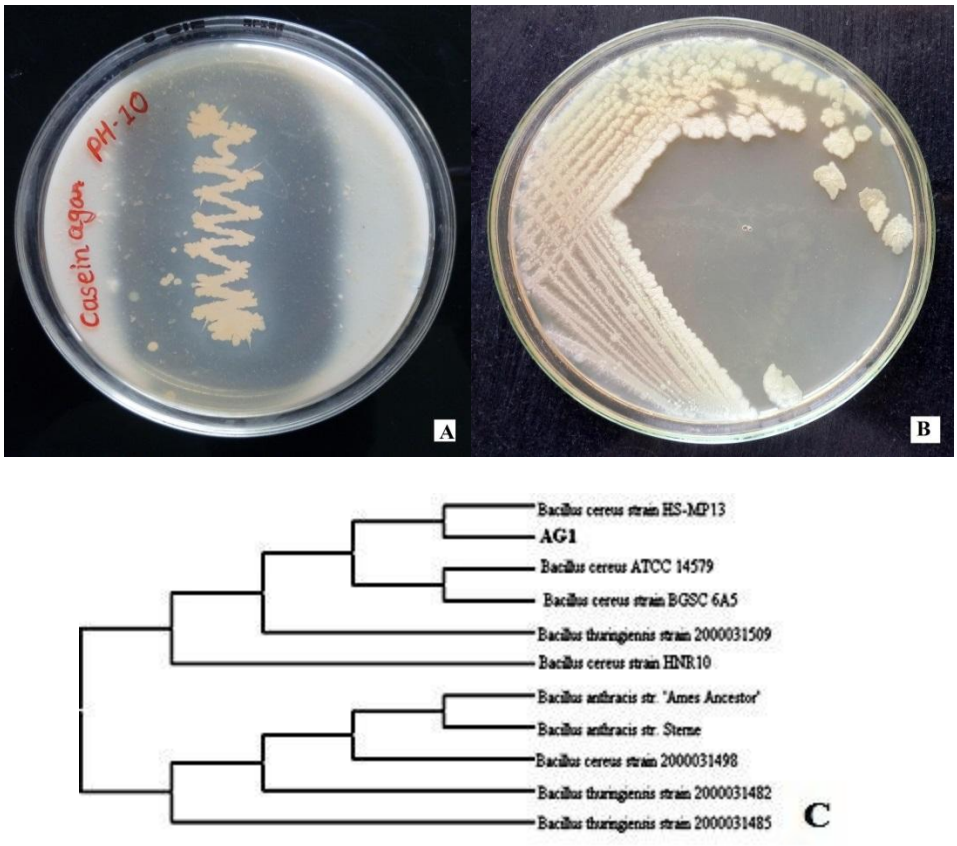

Fig.2 Time course study of alkaline protease production by *Bacillus cereus* AG1

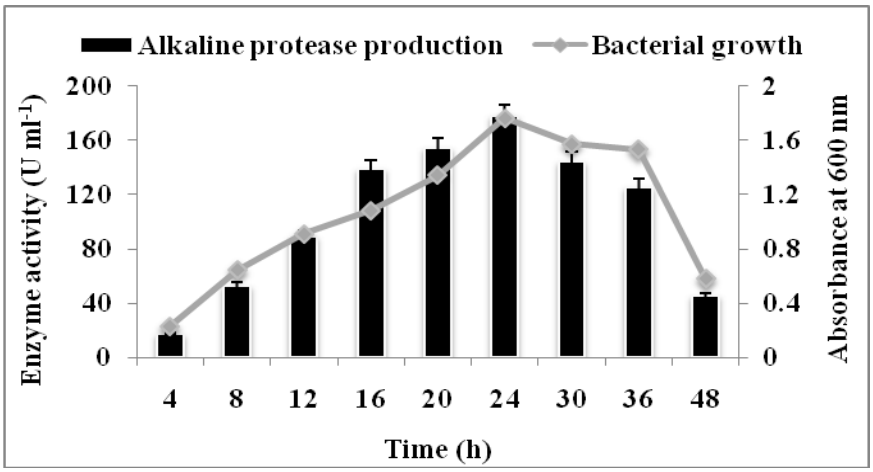

Fig.3 Effect of pH on alkaline protease production by *Bacillus cereus* AG1

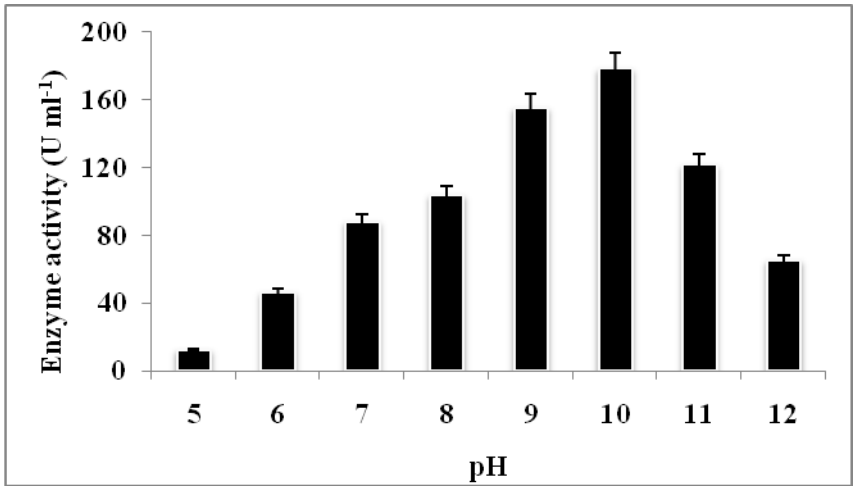

Fig.4 Effect of temperature on alkaline protease production by *Bacillus cereus* AG1

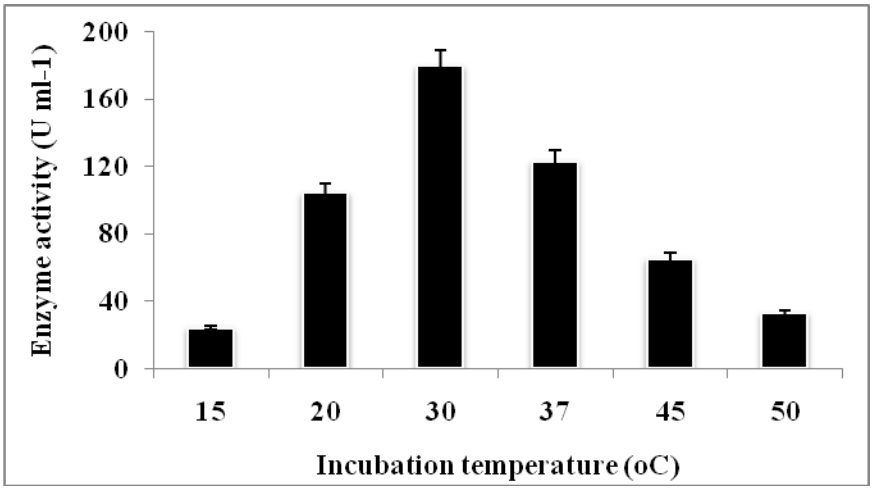

Fig.5 Effect of pH on alkaline protease activity

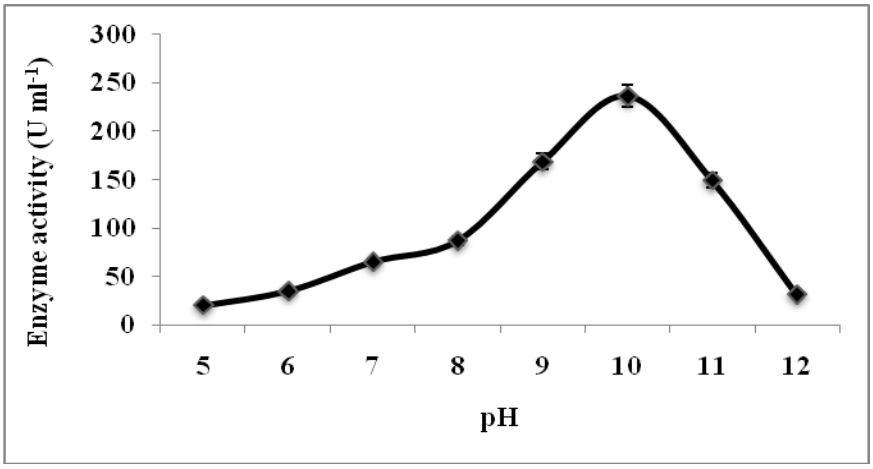

Fig.6 Effect of temperature on enzyme activity

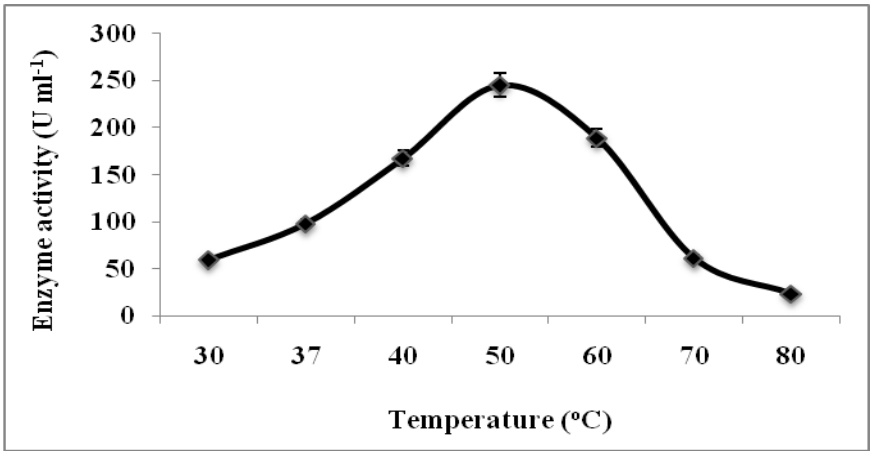

Fig.7 Double reciprocal plot on effect of substrate concentration on alkaline protease activity

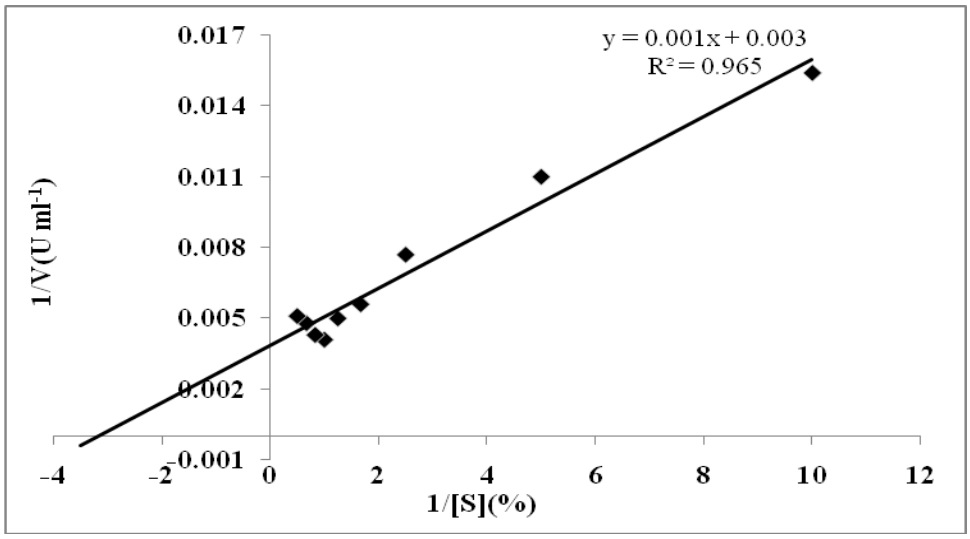

Table.1 Effect of carbon source on alkaline protease production by *Bacillus cereus* AG1

Carbon source	Enzyme activity (U ml^{-1})
Sucrose	70±2.1
Fructose	238±2.5
Glucose	190±1.9
Xylose	45±1.7
Maltose	76±1.6
Mannitol	35±2.1
Na-acetate	28±0.9
Glycerol	43±2.1
Lactose	57±1.8

Table.2 Effect of nitrogen source on alkaline protease production by *Bacillus cereus* AG1

Nitrogen source[a]	Enzyme activity (U ml^{-1})
Beef extract	74±2.4
Yeast extract	92±2.1
Peptone	131±1.9
Beef extract+Yeast extract+Peptone	246±2.5
Gelatin	70±1.5
Urea	86±2.1
Ammonium chloride	58±1.7
Ammonium dihydrogen phosphate	34±2.2
Sodium nitrate	35±2.1
Ammonium sulphate	45±1.4
Potassium nitrate	24±1.8

[a] concentration of fructose was 1.0% (w/v) as carbon source

Table.3 Comparison of steps of partial purification of alkaline protease

Purification steps	Total recovery		Specific activity	Purification fold	% Yield
	Enzyme (Unit)	Protein (mg)			
Crude Enzyme	16160	684	23.63	-	-
80% ammonium sulfate precipitation	11120	315	35.30	1.49	**68.81**
Dialysis	**9721**	**134**	**72.54**	**3.07**	**60.15**

Table.4 Effect of metal ions and inhibitors on alkaline protease activity

	Enzyme activity (U/ml)	Relative activity (%)
Control	252±2.5	**100**
Metal ions		
Ca^{+2}	292±2.4	**115.87**
Hg^{+2}	80±1.5	**31.74**
Na^{+}	163±2.2	**64.68**
Inhibitors		
β-mercaptoethanol **5.0 mM** **10.0 mM**	 81±2.3 41±1.5	 **32.14** **16.27**
EDTA **5.0 mM** **10.0 mM**	 **101±1.7** **0.0**	 **40.08** **0.0**

Table.5 Compatibility of alkaline protease from *Bacillus cereus*AG1with commercial detergents in the presence of glycine and $CaCl_2$

Relative Residual Alkaline Protease Activity (%)						
Time (min)	Control	Arial	Tide	Nirma	Wheel	Local made
0	100	100	100	100	100	100
10	89.5	63.14	86.12	38.55	67.63	23.03
20	84.6	51.94	73.6	30.17	60.26	20.83
30	79.1	42.75	66.13	26.96	56.34	15.94
60	77.4	30.94	58.79	17.00	47.75	15.02
90	69.4	27.01	51.10	10.31	30.80	13.40
120	67.4	24.43	46.60	08.02	28.36	06.05
180	65.0	20.04	52.42	05.16	24.93	05.35

The specific activity obtained after dialysis was 72.54 with 3.07 purification fold. Comparative results of purification of alkaline protease by *Bacillus cereus*AG1 are depicted in table 3.

Characterization of partially purified alkaline protease

Effect of pH on enzyme activity

The partially purified alkaline protease was incubated at different pH values ranging from 5.0-12.0. The enzyme exhibited significant stability and activity in pH range 8.0-11.0 with maximum activity at pH 10.0 (236±2.6 U ml^{-1}) (Fig. 5). The enzyme retained 63.14% activity at pH 11.0. Nevertheless, alkaline protease activities at pH 5.0 and 12.0 were found to be 8.47% and 13.14% respectively. The results thus demonstrated pH dependent enzyme activity. Similar findings were reported by Gomaa (2013), wherein alkaline protease produced by *Bacillus pumillus* exhibited maximum activity at pH 10.0.

Effect of temperature on enzyme activity

In order to evaluate the effect of temperature the partially purified alkaline protease, the enzyme was incubated under different temperatures (30-80^{o}C) and residual activity was determined. The alkaline protease derived from *Bacillus cereus* AG1 was found to be active in broad range of temperature. Results in figure 6 showed that maximum enzyme activity (245±3.1 U ml^{-1}) was obtained at 50^{o}C. At 60^{o}C temperature 77.14 % enzyme activity was observed, which is considerably significant. However, 24.01% and 09.39% enzyme activity was obtained at 70^{o}C and 80^{o}C respectively. The alkaline proteases with higher thermostability are in greater demand for industrial applications (Haddar *et al.*, 2009). Our results are in good accordance with previously reported results of the researchers (Uchida *et al.*, 2004; Yossan *et al.*, 2006).

Substrates specificity of alkaline protease

Three substrates casein, gelatin and BSA were tested for enzyme specificity; maximum activity was obtained with casein (251±2.4 U ml^{-1}). Conversely, least significant enzyme activities were found with gelatin (69±2.2 U ml^{-1}) and BSA (55±2.6 U ml^{-1}). The effect of casein concentration on enzyme activity was determined in the range of 0.1-2.0% (w/v). In our experiment a typical Monod-type profile was observed as the enzyme activity increased from 65 to 248 U ml^{-1} with an increase in casein concentration from 0.1 to 1.0 %.

Under experimental conditions, the correlation between enzyme activity (E_a) and casein concentration ([S])was obtained by Michaelis-Menten double reciprocal model ($E_a$max[S]/(Km+[S]). The values of an apparent maximum enzyme activity ($E_a$max) and Michael is constant (Km) were 250 U ml^{-1} and 0.28% respectively (Fig. 7). Further increase in casein concentration to 2.0% resulted into lowering the alkaline protease activity.

Effect of metal ions and inhibitors on enzyme activity

The alkaline protease activity was determined by incubating partially purified enzyme with different metal ions at 1.0 mM concentration. As depicted in table 4 that enzyme activity was inhibited 31.74% and 64.68% in the presence of Na^{+} and Hg^{+2} respectively. It is noteworthy that the stimulation of alkaline protease activity was evident with Ca^{+2}.The enhancing effect of Ca^{2+} on alkaline protease activity has been well reported (Kumar, 2002). This enhancement in activity may be attributed to the ability of metal ions to protect the enzyme against thermal denaturation and maintain its active conformation (Kumar and Takagi, 1999).

The partially purified alkaline protease from *Bacillus cereus* AG1 was completely inhibited by 10.0 mM EDTA. However, 59.92% enzyme activity was retained in the presence of 5.0 mM EDTA after 3 h incubation. β- mercaptoethanol inhibited the enzyme activity 67.86% and 83.73% at 5.0 and 10.0 mM respectively (Table 4). The detergents contain high amount of chelating agents functioning as water softeners and also assist in stain removal. Therefore, the stability of alkaline protease in the presence of EDTA is of great significance to use as detergent additive (Qasim and Rani, 2003).

Detergent compatibility

The stability of partially purified alkaline protease was performed in the presence of commercial detergents. The enzyme from *Bacillus cereus*AG1displayed varying degree of stability and compatibility with various detergents at 50°C in the presence of $CaCl_2$ and glycine as stabilizers. The alkaline protease retained 52.42% activity with Tide followed by Wheel (24.93%) and Arial (20.04%) after 3 h incubation at 50^{o}C (Table 5). Nevertheless, significant inhibition of enzyme activity was observed with Nirma and Local made detergent. Beside pH, a good detergent protease is expected to be stable in the presence of commercial detergents (Banerjee *et al.*, 1999). Adinarayana *et al.*, 2003 reported activity and compatibility of alkaline protease from *Bacillus subtilis* PE-11 with commercial detergents in the presence of $CaCl_2$ and glycine.

The present study depicts the ability of soil isolate *Bacillus cereus* AG1 for the production of alkaline protease under submerged condition. Optimization of physico-chemical parameters showed 1.39 times higher production of the enzyme. The partially purified alkaline protease displayed significant pH and temperature stability. The presence of Ca^{2+} enhanced enzyme activity. The detergent compatibility suggests the feasibility of alkaline protease from *Bacillus cereus* AG1 for commercial preparation of ecofriendly cleaning formulations.

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