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Research Paper

Optimization and characterization of alkaline protease and carboxymethyl-cellulase produced by *Bacillus pumillus* grown on *Ficus nitida* wastes

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Abstract

The potentiality of 23 bacterial isolates to produce alkaline protease and carboxymethyl-cellulase (CMCase) on *Ficus nitida* wastes was investigated. *Bacillus pumillus* ATCC7061 was selected as the most potent bacterial strain for the production of both enzymes. It was found that the optimum production of protease and CMCase were recorded at 30 °C, 5% *Ficus nitida* leaves and incubation period of 72 h. The best nitrogen sources for protease and CMCase production were yeast extract and casein, respectively. Also maximum protease and CMCase production were reported at pH 9 and pH 10, respectively. The enzymes possessed a good stability over a pH range of 8-10, expressed their maximum activities at pH10 and temperature range of 30-50 °C, expressed their maximum activities at 50 °C. Ions of Hg^{2+}, Fe2+ and Ag^{+} showed a stimulatory effect on protease activity and ions of Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+} and Ag^{+} caused enhancement of CMCase activity. The enzymes were stable not only towards the nonionic surfactants like Triton X-100 and Tween 80 but also the strong anionic surfactant, SDS. Moreover, the enzymes were not significantly inhibited by EDTA or cystein. Concerning biotechnological applications, the enzymes retained (51-97%) of their initial activities upon incubation in the presence of commercials detergents for 1 h. The potential use of the produced enzymes in the degradation of human hair and cotton fabric samples were also assessed.

Key words: alkaliphillic, protease, carboxymethylcellulase, production, characterization.

Introduction

Soda lakes represent the major types of naturally occurring highly alkaline environments in which the indigenous microflora is subjected to number of extreme ecological pressures. They represent the most stable high pH environments on earth, where large amounts of carbonate minerals can generate pH values higher than 11.5 (Grant and Jones, 2000; Jones *et al.*, 1998). Soda lakes are widely distributed throughout the world. One of those environmental niches which have not been studied in details is the Wadi El-Natrun soda lakes in northern Egypt. The features of Wadi El-Natrun area created an ecosystem which considers as rich source for isolation of different extremophiles, including alkaliphillic microorganisms.

Alkaliphiles are defined as organisms that grow optimally at alkaline pH, with pH optima for growth being in excess of pH 8 (usually between 9 and 10), and some being capable of growing at pH higher than 11 (Grant and Jones, 2000; Horikoshi, 1999). Alkaliphillic bacteria are reported to be a rich source of alkaline active enzymes *e.g.* amylase, protease, cellulase, xylanase, and other enzymes which have numerous applications in industrial processes (Horikoshi, 1991).

It has been established that there are three main types of enzymes found in the cellulase system that can degrade cellulose: exo-β-1,4-glucanase, EC 3.2.1.74; endo-β-1,4-glucanase, EC 3.2.1.4 (Carboxymethyl cellulase) and β-glucosidase, EC 3.2.1.21. The endoglucanases act internally on the chain of cellulose cleaving β-linked bond liberating nonreducing ends, and exoglucanases remove cellobiose from this non-reducing end of cellulose chain. Finally, β-glucosidase completes the saccharification by

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splitting cellobiose and small cellooligosaccharides to glucose molecule (Silva *et al.*, 2005). Potential applications of cellulases are in food, animal feed, textile, fuel, chemical industries, paper and pulp industry, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment (Tarek and Nagwa, 2007).

Alkaline protease has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications *e.g.* in the detergents industry as additives, food processing, tanning, waste treatment, textile industry in the process of dehairing and leather processing and also have application in silver recovery from photographic plates. Furthermore, they are used in pharmaceuticals and medical diagnosis (Gupta *et al.*, 2002; Joo *et al.*, 2003).

Agricultural residues such as grasses, tree wastes and many other green plants whose disposal is considered as an environmental problem as they have been accumulating or used inefficiently due to the high cost of their utilization processes (Lee *et al.*, 2006). *Ficus nitida* L. (Moraceace) is a widely cultivated ornamental tree in Egypt. It yields a vast amount of wastes yearly either from fallen leaves or as a result of continuous shaping and pruning. *Ficus* wastes are highly nutritious, containing considerable amounts of celluloses, proteins and trace elements (Kitajima and Kimizuka,1998).

The present study aimed at studying the optimum conditions for production of alkaline protease and CMCase enzymes by *Bacillus pumilus* ATCC7061, isolated from Wadi El-Natrun soda lakes, grown on low cost substrate (*Ficus nitida* wastes). Furthermore, characterization and application of the produced enzymes were also studied.

Materials and Methods

Isolation of alkaliphillic bacteria

Four different soil samples were collected from different localities of Wadi El-Nartoun in northern Egypt. These were Dawood, El-Bida, El-Hamra and Bani Salama. Isolation of alkaliphillic bacteria was carried out using alkaline agar medium of Horikoshi (1990). It contained 1% glucose, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 1% Na_2CO_3 and 1.5% agar, pH 10.5. Aliquots (100 µL) of different dilutions of soil suspensions samples were plated and incubated at 30 °C for three days. According to the morphological characteristics of different colonies on agar plates, inocula from these grown colonies were transferred into replicates of slants containing the same specific media. Purified isolates were maintained on agar slants of the same medium at 4 °C and was subcultured at monthly intervals.

Screening of protease and CMCase enzymes production

Purified colonies were transferred to skim milk agar plates to be screened for protease production. The medium contained peptone (0.1%), NaCl (0.5%), agar (2.0%), and skim milk (10%) (Ellaiah *et al.*, 2002a). The appearance of clear zones around the colonies as a result of casein hydrolysis was taken as an indication of protease production. The strains showing proteolytic activities were screened for CMCase production. A preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye method of Ariffin *et al.* (2006). The bacteria were grown on CMC agar containing (g/L): KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.5, NaCl 0.5, $FeSO_4.7H_2O$ 0.01, $MnSO_4.H_2O$ 0.01, NH_4NO_3 0.3, CMC 10.0, Agar 20.0. The formation of a clear zone of hydrolysis indicated cellulose degradation. The strain showed the highest production of protease and CMCase enzymes was selected for further experimental studies.

Strain identification

Strain Alk9 which was the highest protease and CMCase producer was identified by 16S rDNA sequence. Comparisons of the sequence between different species suggest the degree to which they are related to each other. This was done by constructing phylogenetic tree using neighbour-joining (N-J) method (Ariffin *et al.*, 2006).

Production of alkaline protease and CMCase enzymes

Production of protease by *Bacillus pumilus* ATCC7061 was carried out in a medium containing the following (g/L): glucose.1.0, yeast extract, 0.5, $CaCl_2$.0.1, K_2HPO_4, 0.5 and $MgSO_4$, 0.1 (Ul-Qadar *et al.*, 2009). The peptone content of the original medium was replaced with washed and ground dry leaves of *Ficus nitida* in a concentration of 10 g/L. Production of CMCase was carried out in a medium containing the following (g/L): KH_2PO_4 1.0, K_2HPO_4 1.145, $MgSO_4.7H_2O$ 0.4, $(NH_4)_2SO_4$ 5.0 $CaCl_2.2H_2O$ 0.05 and $FeSO_4.7H_2O$ 0.0012 (Ariffin *et al.*, 2006). *Ficus nitida* leaves at concentration of 10 g/L were used as carbon source instead of CMC.

Erlenmeyer conical flasks of 500 mL capacity containing 100 mL of the modified media adjusted at pH 10 were inoculated with 1.0 mL spore suspension of the strain (adjusted to 1.0 OD_{600}) and incubated at 30 °C in a rotary incubator (150 rpm) for 72 h. The culture fluids were centrifuged at 10000 xg for 20 min and the clear cell free supernatants were regarded as crude enzyme supernatants.

Protease and CMCase activities assay

Proteases activity was determined by a slightly modified method of Yang *et al.* (2000). The reaction mixture containing 1 mL of 1.0% casein solution in 0.05 M Glycine-NaOH buffer having pH 10 and 1 mL of a given enzyme solution were incubated at 40 °C for 30 min and the reaction was then stopped with 3 mL of 10% trichloroacetic acid (TCA), mixed well and the non-hydrolyzed casein was precipitated by centrifugation at 10,000 xg for 10 min. The

supernatant was utilized for protease determination according to the method established by Lowry *et al.* (1951) using a standard curve prepared with tyrosine. One unit of alkaline protease activity was expressed as the amount of the enzyme that released 1µ mole of tyrosine per minute under the assay conditions. CMCase activity was assayed using a modified method described by Wood and Bhat (1998) with some modifications. Briefly, 0.2 mL of culture filtrate was added to 1.8 mL of 1% CMC prepared in Glycine-NaOH buffer (pH 10) in a test tube and incubated at 40 °C for 30 min. The reaction was terminated by adding 3.0 mL of dinitrosalicylic acid (DNS) reagent and by subsequently placing the reagent tubes in water bath at 100 °C for 15 min. One milliliter of Rochelle salt solution (40%) was then added to stabilize the color. The absorbance was recorded at 575 nm against the blank (of 0.05 M sodium citrate buffer). One unit of CMCase activity was expressed as 1 µmole of glucose liberated per ml enzyme per minute.

Biomass yield

Bacterial biomass was determined by measuring the absorbance at 600 nm.

Factors affecting the enzymes production

pH and temperature

In order to investigate the influence of pH on enzymes production, *Bacillus pumilus* ATCC7061 was grown at different pH values (7.0-11.0). Similarly, the influence of temperature was investigated by varying the incubation temperature (20-40 °C) at optimum pH, keeping the other parameters also constant.

Ficus nitida leaves concentration

Different concentrations (0.5-6%) of *Ficus nitida* leaves were added separately to the production media of protease and CMCase enzymes.

Nitrogen sources

Yeast extract and ammonium sulphate (control) in case of protease and CMCase production media, respectively were replaced at equivalent nitrogen concentration by different nitrogen sources. Five inorganic nitrogen sources (ammonium chloride, ammonium nitrate, ammonium sulphate , potassium nitrate and sodium nitrate) and five organic nitrogen sources (ammonium acetate, casein, peptone, urea and yeast extract) were separately supplemented to the medium. The same cultural conditions as previously stated were adopted.

Salts

Different K_2HPO_4 concentrations (0, 0.25, 0.5, 1.0 and 1.5 g/L) were added separately in protease production medium. On the other hand, CMCase production medium contained (1.0+1.14) g/L of KH_2PO_4+K_2HPO_4 was replaced by different concentrations (0 + 0, 0.5 + 0.64, 1.5 + 1.64 and 2.0:2.14 g/L). These concentrations were added separately to the modified medium. Also, different concentrations of $MgSO_4$ (0.0-0.8 g/L) were added separately to the modified protease and CMCase production media to determine the most promising concentration for enzymes production.

Incubation period

After optimizing all the nutritional and environmental factors described above, the protease and CMCase production media were cultivated for 5 days. During cultivation, a sample of culture broth was withdrawn periodically (every 24 h) for enzymes activities determination as mentioned before.

Characterization of protease and CMCase

Effect of pH on enzymes activity and stability

Effect of pH on protease and CMCase activities were determined by assaying the enzyme activity as described above at different pH values ranging from 7.0 to 12.0, using the following buffer systems: 0.05 M of phosphate (pH 7), tris-HCl (pH 8-9) and glycine-NaOH (pH 10-12). The pH stability of the enzymes was investigated in the same pH range and incubated at 40 °C for 1 h. Afterwards, aliquots of the mixtures were taken to measure the residual protease activity (%) with respect to the control, under standard assay conditions.

Effect of temperature on enzymes activity and stability

Effect of temperature on enzymes activities were determined by incubating the reaction mixture at optimum pH value under different temperature ranging from (30-80 °C). In order to determine the thermostability of the enzymes, experiments were conducted by measuring the residual activity after incubation in the same temperatures range for 60 min under standard assay conditions.

Effect of various metals ions, surfactants and inhibitors on enzymes activity

Effect of metal ions, surfactants and inhibitors on the activity of protease and CMCase enzymes were determined in the presence of metal ions ($K^{+,}$ Na^+, Ag^+, Ca^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, and Zn^{2+}, final concentration of 5 mM), SDS, Triton X-100 and Tween 80 (1%), Ethylenediamine tetraacetic acid (EDTA) and L-cystein (5 mm).The enzymes were pre-incubated with the above-mentioned chemicals for 1 h at 50 °C, afterwards, the relative activity (%) was calculated with respect to the control where the reaction was carried out in the absence of any additive under the optimum assay conditions (Lo *et al.*, 2001).

Compatibility with commercial detergents

Detergents available in the national market, such as Ariel, Persil, Tide and X-tra were used. To simulate washing conditions, these detergents were diluted in distilled

water up to a final concentration of 7.0 mg/mL. The solutions were boiled for 10 min for the inactivation of their enzymes contents already present and cooled. Protease and CMCase enzymes of *B. pumilus* ATCC7061 were then added to the detergents solution in a ratio of (1:1) and incubated for 1 h at 50 °C. The residual activities were then determined. The enzymes activities of control samples (without any detergent) were taken as 100% (George *et al.*, 2001).

Application of protease and CMCase enzymes

Degradation of human hair

Untreated black human hair (1 g) was suspended in 50 mL protease of *Bacillus pumilus* and the reaction mixture was incubated at 40 °C for 1 h. The reaction mixture was cooled and filtered to stop the enzymatic reaction. Human hair was dried and weighed to determine the loss of weight.

Tests on cotton fabric

Cotton fabric sample (1.5 g) was incubated at room temperature with 50 mL CMCase of *Bacillus pumilus* ATCC 7061. After 6 days, the samples were washed in water, dried, and weighed to determine the loss of weight.

Statistical analysis

Results are presented as mean value ± standard deviation (SD).The Microsoft Excel 2003 and SAS 9.1.3 statistical program were used for data analysis.

Results and Discussion

Isolation and screening of alkaliphillic bacteria

Twenty three alkaliphillic bacteria were isolated from four different soil samples collected from different localities of Wadi El-Nartoun in northern Egypt. The isolates exhibited diversity towards gram's reaction, colony and cell morphology including 11 Gram positive rods, 7 Gram positive cocci, 3 Gram negative rods and 2 Gram negative cocci (data not shown). After preliminary investigation of these alkaliphillic bacteria to produce protease and CMCase enzymes, only 12 (52%) isolates were found to be protease producers as detected by clear zones of casein hydrolysis around colonies in skim-milk agar plates. Among these bacterial strains seven strains exhibited evident clear zones around the colonies on CMC agar plates and were recorded as cellulase producers.

It was found that Alk9 isolate yielded the highest protease and CMCase enzymes productivity. In order to identify the strain, the nucleotide sequence of the 16S rDNA of the strain was determined. Phylogenetic tree was constructed by the neighbour-joining (N-J) method based on the 16S rDNA sequences. The results revealed that strain Alk9 exhibited high level of 16S rDNA similarity (99%) with *Bacillus pumilus* ATCC7061.

Factors affecting protease and CMCase production

pH

The results presented in Figure 1 show that production of protease and CMCase by *Bacillus pumilus* ATCC7061 were increased with increasing pH of medium towards alkaline range from neutrality. Maximum enzymes production was at pH 9 (21.45 U/mL) and 10 (15.86 U/mL) for protease and CMCase, respectively. This suggested that the bacterial strain was alkaliphillic in nature and optimum pH range between 9 and 10 for growth and enzymes production is common feature among alkaliphillic organisms (Denizci *et al.*, 2004; Johnvesly and Naik, 2001). It is well known that pH of the culture medium affects the availability of certain metabolic ions and permeability of bacterial cell membranes, which in turn support the cell growth and enzymes production (Ellaiah *et al.*, 2002b).

Temperature

Growth temperature is another critical parameter that needs to be controlled. The incubation temperature is usually determined by considering the sources from which the organisms have been isolated. Maximum production of protease and CMCase enzymes were recorded at 30 °C (Figure 2). At higher temperature, both enzymes production decreased because the enzymes begin to suffer thermal inactivation.

Concentration of Ficus nitida leaves

On studying the ability of *Bacillus pumilus* ATCC7061 to utilize *Ficus nitida* wastes and/or produce alkaline protease and CMCase enzymes, it was found that 5% (w/v) was optimum for both enzymes production (Figure 3). Further increasing in the concentration had an adverse effect on enzymes production. *Ficus nitida* wastes were used in the present study as a sole source of carbon for CMCase production and as a nitrogen source for protease production. Chemical analysis of *Ficus nitida* leaves used

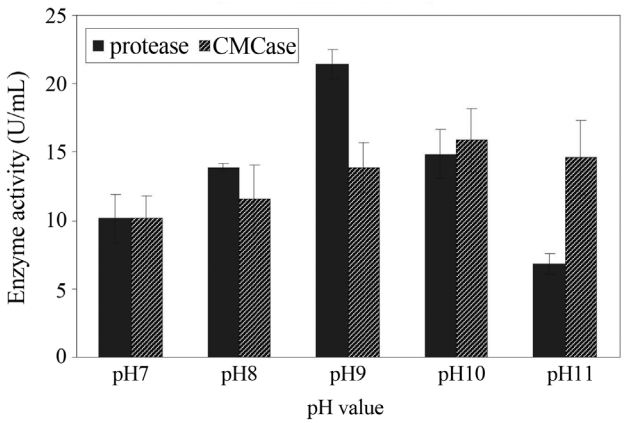

Figure 1 - Effect of different pH values on the production of protease and CMCase by *Bacillus pumilus* ATCC 7061. Results are means of three independent determinations. Bars correspond to standard deviation.

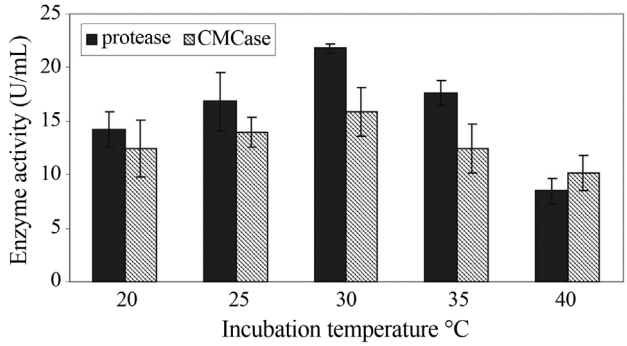

Figure 2 - Effect of incubation temperature on the production of protease and CMCase by *Bacillus pumilus* ATCC 7061. Results are means of three independent determinations. Bars correspond to standard deviation.

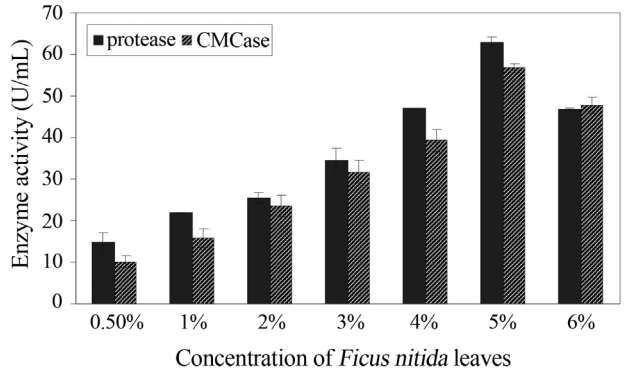

Figure 3 - Effect of different concentrations of *Ficus nitida* leaves on the production of protease and CMCase by *Bacillus pumilus* ATCC 7061. Results are means of three independent determinations. Bars correspond to standard deviation.

in the fermentation medium showed it contained 33.8% cellulose and 6.31% protein. These results showed the efficacy of applying these agricultural wastes as plentiful and cheap media for biomass production that will benefit the utilization of these abundant agricultural wastes and to produce many important substances with economic importance. In this regard, Nizamudeen and Bajaj (2009) previously replaced CMC of the cellulase production medium with alternative substrates such as wheat straw, wheat bran, saw dust or filter paper as alternative approaches for production of low cost enzymes from bacteria.

Nitrogen sources

Nitrogen source has got profound influence on enzyme production as it is the ultimate precursor for protein biosynthesis. Besides, the nitrogen source can also affect the pH of the medium, which in turn may influence the activity and stability of the enzyme (Nizamudeen and Bajaj, 2009). On an equivalent nitrogen basis, yeast extract and ammonium sulphate in the original media of protease and CMCase, respectively were replaced by different inorganic and organic nitrogen sources, one at a time. Results illustrated in (Figure 4) demonstrated that generally, inorganic nitrogen sources proved less favorable towards enzymes secretion. The same result was previously recorded by Gajju *et al.* (1996) who found that organic nitrogen sources better suited to *Bacillus* sp. for enzymes production as organic nitrogen sources being rich in amino acids and short peptides that displayed enzymes production.

The results cited in Figure 4 also showed that the maximum productivity of protease (63.03 U/mL) and CMCase (70.07 U/mL) were recorded in the presence of yeast extract and casein, respectively. Yeast extract is an inexpensive organic source of amino acids, proteins and vitamins. It contains abundant nitrogen compounds as well as many growth factors. Casein also contains some essential amino acids as well as some carbohydrates and the inorganic elements calcium and phosphorus.

Salts

Maximum productivity of protease (63.14 U/mL) and CMCase (93.47 U/mL) were recorded in the presence of 0.5 g/L of K_2HPO_4 and/or KH_2PO_4 (Table 1). Beyond this concentration, both enzymes production decreased drastically. The enzymes activity reduced at higher concentrations presumably due to repression of enzymes synthesis. This effect was quite comparable to the result for alkaline protease production by *Bacillus firmus* in which increased supply of nitrogen and phosphorus stimulated protease synthesis up to certain threshold levels (Moon and Parulekar , 1991). Phosphate plays a vital role as an effector of a large number of enzymatic reactions of primary metabolism, including synthesis of deoxyribonucleic acid and ribonucleic acids (DNA, RNA) and proteins, carbohydrate metabolism, cellular respiration, and control of adenosine triphosphate (ATP) levels.

It is clear from Table 1 that maximum protease (63.14 U/mL) and CMCase (93.47 U/mL) were reported when $MgSO_4$ was added in a concentration of 0.1 and 0.4 g/L, respectively. Any further supplementation had an

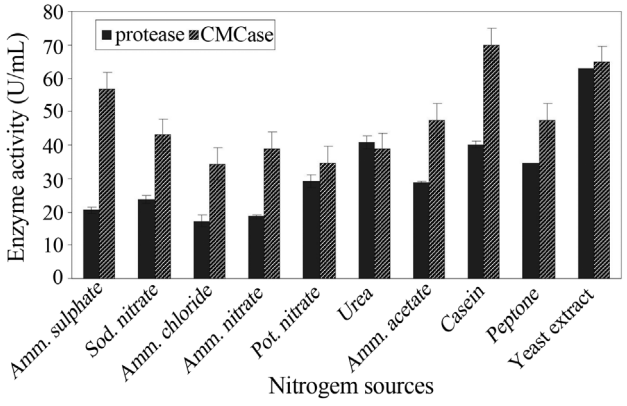

Figure 4 - Effect of different nitrogen sources on the production of protease and CMCase by *Bacillus pumilus* ATCC 7061. Results are means of three independent determinations. Bars correspond to standard deviation.

Table 1 - Effect of some salt concentrations on the production of protease and CMCase by *Bacillus pumilus* ATCC 7061.

Salt conc. (g/L)	Protease activity (U/mL)	Salt conc. (g/L)	CMCase activity (U/mL)
	K_2HPO		K_2HPO_4/ KH_2PO_4
0.00:	53.68 ± 0.01**	0:0:	60.83 ± 0**
0.25:	55.44 ± 0.01**	0.5:0.64:	93.47 ± 0.04^n
0.5:	63.14 ± 0**	1.0:1.14:	70.07 ± 0.01**
1.0:	45.87 ± 0.03*	1.5:1.64:	31.57 ± 0.1^n
1.5:	35.75 ± 0.005^n	2.0:2.14:	14.63 ± 0.01*
	$MgSO_4.7H_2O$		$MgSO_4.7H_2O$
0.05:	53.68 ± 0.1^n	0:	42.19 ± 0.6**
0.1:	63.14 ± 0.01**	0.2:	52.97 ± 0.2^n
0.2:	45.87 ± 0**	0.4:	93.47 ± 0.01*
0.3:	35.75 ± 0.02*	0.6:	65.45 ± 0.01*
0.4:	30.00 ± 0*	0.8:	48.81 ± 0.02*

Data represent the mean of 3 different readings ± standard deviation.
^{n} =Non significant, $p < 0.05$;*=significant, $p \geq 0.01$ and ≤ 0.05; **= highly significant, $p < 0.01$.

inhibitory effect on both enzymes production. This suggested an important role of Mg^{+2} to increase and stabilized the enzymes production (Paliwal *et al.*, 1994).

Incubation period

The results represented in Figure 5 show that cell growth and enzymes production increased linearly with increase in incubation time suggesting that enzyme production was growth associated in nature (Johnvesly *et al.*, 2002 ; Ariffin *et al.*, 2006). Maximum growth and enzymes production were recorded after 72 h of incubation period and this has been reported previously by Heck *et al.* (2002); Amritkar *et al.* (2004).

Partial characterization of the produced enzymes

The description and characterization of the enzymes produced by extremophilic organisms is relevant not only for their participation in the natural cycles in the environment but also for their possible application in industry.

Effect of pH value on enzymes activity and stability

Testing the pH-dependence of enzymes activity reveled that both enzymes possessed activities over a broad pH range (8-12). The results represented in Figures 6 and 7 show that pH 10 was optimum for protease (63.58 U/mL) and CMCase (95.32 U/mL) and there was a sharp decrease in activity at pH 7. This optimum pH value is identical to that found for endogluconase from *Bacillus* sp. (Endo *et al.*, 2001).

As the pH value diverged from the optimum level, the efficient functioning of the enzyme affected and this could be ascribed to decreased saturation of the enzyme with substrate due to a decreased affinity and/or due to the effect of

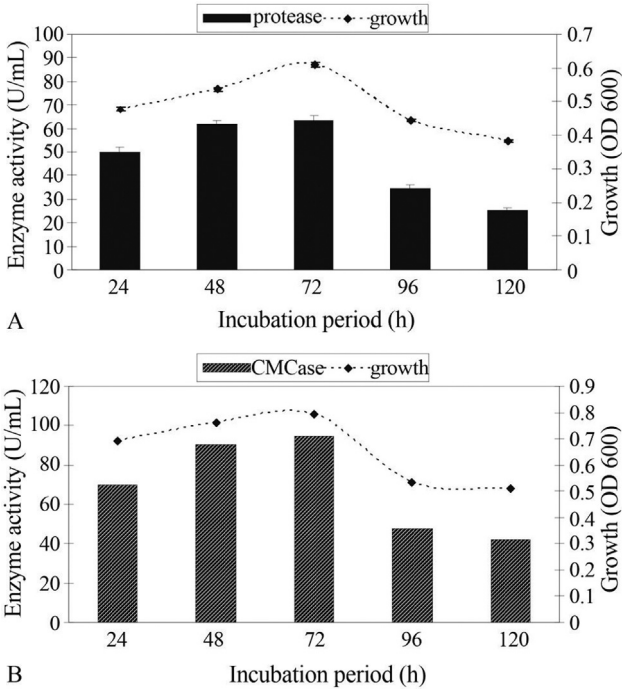

Figure 5 - Effect of different incubation period on growth and the production of protease (A), growth and the production of CMCase (B) by *Bacillus pumilus* ATCC 7061. Results are means of three independent determinations. Bars correspond to standard deviation.

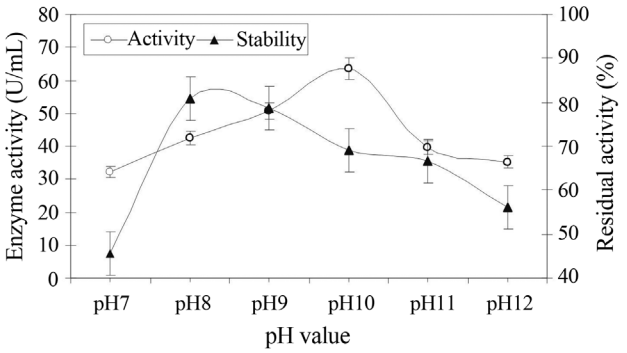

Figure 6 - Activity and stability of the protease produced by *Bacillus pumilus* ATCC7061 as a function of pH value. Results are means of three independent determinations. Bars correspond to standard deviation.

pH on the stability of the enzyme (Dixon and Webb, 1979). In this study, testing pH stability of the protease and CMCase produced by *Bacillus pumilus* ATCC7061 revealed that maximum stability of protease and CMCase enzymes were observed at pH8 and pH9, they retained 80.79 and 93.21% of their original activities, respectively after incubation of the enzymes for one hour at 40 °C within this pH range (Figures 6 and 7). Taking into account such considerations and the well known fact that many industrial processes are operated at pH extremes (either acidic or alkaline) therefore, the industrially important enzymes must be capable of withstanding such harsh and hostile condi-

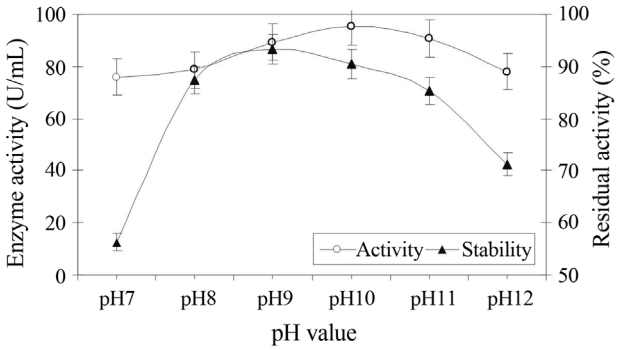

Figure 7 - Activity and stability of the CMCase produced by *Bacillus pumilus* ATCC7061 as a function of pH value. Results are means of three independent determinations. Bars correspond to standard deviation.

tions for prolonged periods or at least during the process time.

Effect of temperature on enzymes activity and stability

Temperature is a critical factor for maximum enzyme activity and it is a prerequisite for industrial enzymes to be active and stable at higher temperature. Results cited in Figures 8 and 9 show that optimum activities were recorded at 50 °C (64.90 and 106.10 U/mL) for protease and CMCase, respectively. The activities reduced to

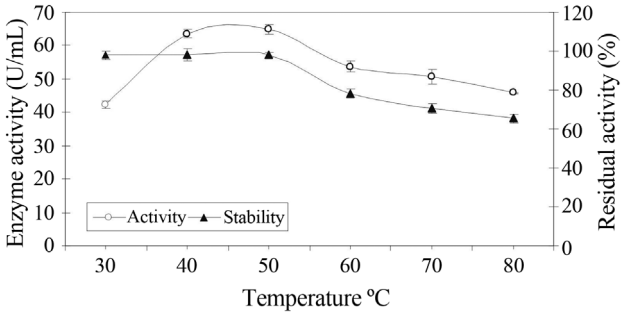

Figure 8 - Thermal activity and stability of the protease produced by *Bacillus pumilus* ATCC 7061. Results are means of three independent determinations. Bars correspond to standard deviation.

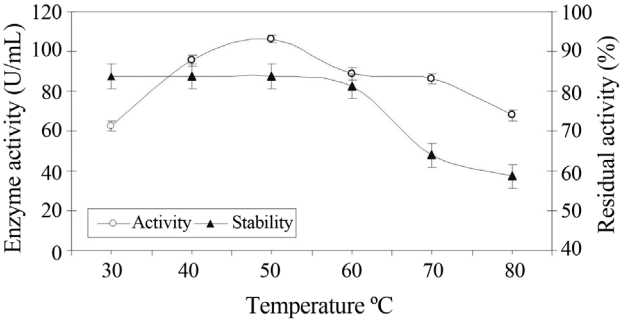

Figure 9 - Thermal activity and stability of CMCase produced by *Bacillus pumilus* ATCC 7061. Results are means of three independent determinations. Bars correspond to standard deviation.

50.60 (U/mL) for protease and 86.24 (U/mL) for CMCase at 70 °C. The enzymes were completely inactivated at 80 °C. Similarly Kumar (2002) found maximum activity of protease produced by *B. pumilus* was at 50-55 °C.

For analysis of thermal stability, enzymes were incubated for 1 h in glycine-NaOH buffer (pH 10.0) at different temperatures (30-80 °C). The results illustrated in Figures 8 and 9 revealed that both enzymes showed good activities over a temperature range of 30-60 °C. However, the enzymes were rapidly inactivated and retained only 70.67 and 64.00% for protease and CMCase, respectively after 1 h incubation at 70 °C. There were sharp decreases in the enzymes activity with further increase in temperature due to the denaturation of the enzyme proteins (Dixon and Webb, 1979).

These findings suggest that the protease and CMCase secreted by *Bacillus pumilus* ATCC7061 have highly and stable alkaline properties with moderate heat stability which indicated to be good additives for enzyme-based detergent applications as they can withstand and work at elevated pH and temperatures during process conditions (Bischoff *et al.*, 2006).

Effect of some metals ions, surfactants and inhibitors on enzymes activity

Effect of some metals ions, surfactants and some inhibitors on activity of protease and CMCase were investigated and the data are presented in Table 2. While Hg^{2+}, Fe^{2+} and Ag^{+} enhanced protease activity slightly, metals caused enhancement of CMCase were Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+} and Ag^{+}. Paliwal *et al.* (1994) previously stated that the metal ions apparently protected the enzymes against thermal denaturation and played a key role to continue the active conformation of the enzymes at high temperatures. Moreover, Mansfield *et al.* (1998) suggested that Ca^{2+} ions have been reported to be required for enhancing the substrate binding affinity of the enzyme and stabilizing the conformation of the catalytic site.

While Kumar (2002) reported that *B. pumilus* alkaline protease lost 22% activity on treatment with 0.1% SDS for 1 h. Protease and CMCase enzymes from *Bacillus pumilius* were stable not only towards the nonionic surfactants like Triton X-100 and Tween 80 but also the strong anionic surfactant, SDS. Moreover, the enzymes were not significantly inhibited with the specific inhibitor EDTA or with cystein. They retained 88-95% of their activities by adding these inhibitors, suggesting that metal cofactors are not required for enzymes activities. This property of the enzymes was very useful for application as detergent additive (Beg and Gupta, 2003). These results were in accordance with Aygan and Arikan (2008) who reported that the endoglucanase enzyme produced by *Bacillus* sp. C14 was resistant to chelating agent EDTA and SDS by preserving original enzyme activity around 93 and 81%, respectively.

Table 2 - Effect of different metals ions, surfactants and inhibitors on the activity of alkaline protease and CMCase enzymes by *Bacillus pumilus* ATCC 7061.

Metals ions, surfactants and inhibitors	Relative activity (%)	
	Protease	CMCase
Control (no addition)	100	100
NaCl	98.8 ± 5.39**	93.7 ± 3.20**
KCl	96.1 ± 2.62^{n}	85.3 ± 2.07**
$AgNO_3$	102.5 ± 2.01^{n}	103.4 ± 5.54**
$MgCl_2$	87.9 ± 0.32**	102.6 ± 2.00**
$CaCl_2$	95.2 ± 2.14**	102.1 ± 4.30*
$CuSO_4$	94.7 ± 6.46*	109.2 ± 0**
$ZnSO_4$	98.8 ± 4.08^{n}	87.9 ± 1.53**
$FeSO_4$	105.5 ± 3.16**	125.2 ± 3.25^{n}
HgI_2	102.2 ± 1.45**	88.96 ± 1.66**
SDS	92.03 ± 2.62^{n}	93.3 ± 2.00**
Triton X-100	95.9 ± 5.13**	82.4 ± 3.00*
Tween 80	89.1 ± 1.48*	94.9 ± 1.38*
EDTA	92.03 ± 2.48**	91.1 ± 0.21^{n}
L-cystein	95.9 ± 0.07**	88.67 ± 0.07

Data represent the mean of 3 different readings ± standard deviation.
^{n} = Non significant, $p < 0.05$;*=significant, $p \geq 0.01$ and ≤ 0.05; **= highly significant, $p < 0.01$.

Compatibility with Detergents

Enzymes activity and stability in presence of some available commercial detergents was studied with a view to exploit the enzymes in detergent industry. Protease maintained 78.81, 97.9, 94.06 and 92.71% of residual activity in Aerial, Tide, Persil and Xtra, respectively, after 1 h incubation, at 50 °C. Whereas, CMCase retained 78.66, 72.13, 56.02 and 51.81% in the mentioned detergents (Figure 10). CMCase produced by an alkalithermophillic actinomycete kept a residual activity of 85, 72 and 66% when tested with the detergents (George *et al.*, 2001). Also, Madan *et al.* (2002) studied the compatibility of alkaline protease from *Bacillus polymyxa*, it retained 20-84.5% of its activity in various detergents. Comparing these results, the alkaline protease and CMCase produced by *B. pumilus* ATCC7061 were significantly more stable in commercial detergents.

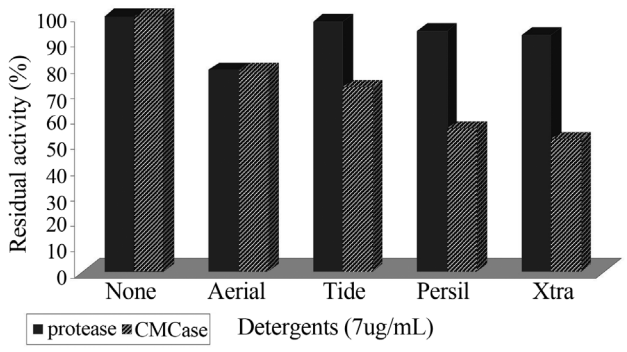

Figure 10 - Compatibility of alkaline protease and CMCase activities from *B. pumilus* ATCC7061 with commercial detergents.

Application of protease and CMCase

An application for protease enzyme was degradation of human hair. The present results showed that the human hair degraded by 50%. It was appeared to be potentially useful in washing powder. Alkaline protease speeds up the process of dehairing, because the alkaline conditions enabled the swelling of hair follicle protein and allowed easy removal of the hair. Horikoshi (1999) reported that alkaline enzymes had been used in the hide dehairing process, where dehairing was carried out at pH values between 8 and 10. Proteases are used for selective hydrolysis of nonfibrillar proteins of the skin and for removal of non fibrillar proteins such as albumins and globulins. The purpose of soaking is to swell the hide, and this step was performed with alkali (Godfrey and West, 1996). In addition, an application of CMCase was degradation of cotton fibers. The experiment conducted aiming at the application in textile industry, measuring the loss of weigh of a cotton fabric. CMCase had shown a loss of weight by 40%. This can indicate a possible application of CMCase from *B. pumilus* in stonewashing and biopolishing.

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