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High salt-tolerant protease from a potential biocontrol agent *Bacillus pumilus* M3-16

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Abstract - In this paper, we investigate the characterization and evaluation of the antifungal protease activity from a halotolerant strain M3-16 of *Bacillus pumilus*, earlier isolated from a shallow salt lake in Tunisia. Protease enzyme was highly induced by the pathogen tested *in vitro* (27.4 U/ml). This is the first report on high salt-tolerant protease from *B. pumilus*, since it was active at high salinity (from 5 to 30% NaCl, w/v) as well as in the absence of salinity. This enzyme showed optimal activity at 60 °C and pH 8. At 80 °C and 30 min, the enzyme retained up to 91% and it showed stability over a wide pH range (from pH 5 to 11). The enzyme was found to be monomer with an estimated molecular mass of 31 kDa. The amino acid sequence showed high similarity (94%) to ATP-dependent protease from *B. pumilus* strain ATCC 7061. Thus, our alkaline thermostable and high salt-tolerant protease induced by a phytopathogenic fungus, could be useful for application in diverse areas such as biotechnology alimentary and agronomy industries.

Key words: *Bacillus pumilus;* halotolerant*;* antifungal protease*;* biocontrol agent.

INTRODUCTION

Enzymes have attracted attention from researchers all over the world because of the wide range of physiological, analytical and industrial applications, especially, from microorganisms, because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation. It is known that, proteases have found a wide range of applications in various industries such as food, pharmaceutical, cosmetics, etc. and have been widely commercialised by various companies throughout the world. Although the production of these enzymes has been improved significantly by the utilisation of hyper-producing strains of fungi as well as bacteria and genetically modified microbes, the present enzymes toolbox is not sufficient to meet most of the industrial demands. Therefore, efforts are still being done to find newer sources of enzymes, better production techniques and novel applications of these enzymes in unexplored fields (Sumantha *et al*., 2006). In view of these restrictions, researchers have diverted their attention to isolation and characterization of enzymes from extremophiles. Extremophiles are valuable sources of novel enzymes. These extremophiles present a commercial potential for various industries (Rothschild and Mancinelli, 2001). In bibliography, the number of proteases has been characterized specially from alkaliphilic bacteria but similar explorations and studies from halophilic and halotolerant bacteria are further restricted (Thumar and Singh, 2007).

The present work is a continuity of the project focused on discovering new microorganisms that could be used as biological control agents against grey mould in strawberry, isolated from extreme saline soil from Tunisia, able to suppress or at least to reduce grey mould disease on strawberries. The research also aimed to identify the mode of action of the most successful isolates as this information may help to optimise their biocontrol efficiency in the field (Sadfi-Zouaoui *et al*., 2008; Essghaier *et al*., 2009). Understanding the mechanisms involved in biological control may enable enhancing control efficacy and reducing the inconsistency and variability.

Therefore, the objective of this work was to evaluate and characterize the proteolytic activity from an halotolerant antagonistic bacterium, *Bacillus pumilus* strain M3-16, earlier isolated from Tunisian Sebkha and selected for its antagonistic activity against *Botrytis cinerea*, on strawberries (Essghaier *et al*., 2009).

MATERIALS AND METHODS

Screening and taxonomic analysis of strain M3-16. A newly halotolerant *Bacillus pumilus* strain M3-16 was isolated from a shallow salt lake in Tunisia (Essghaier *et al*., 2009). The nucleotide sequence of 16S rRNA was previously reported and has

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been deposited in the GenBank data base under the accession number EU435356. This strain was selected as a highly effective antagonist towards *B. cinerea*, the causal agent of grey mould disease on strawberries as well as for each highly enzymatic production (Essghaier *et al*., 2009).

Media composition and culture conditions for protease induction. The investigation of the growth culture conditions for the induction of protease by *B. pumilus* strain M3-16 was carried out in a basal medium Tryptone Glucose Yeast-extract (TGY, medium 1-M1) containing 5 g/l tryptone, 1 g/l glucose, 5 g/l yeast extract, 1 g/l K_2HPO_4 at pH 7 and in other four media: medium 2 (M2), TGY with glucose at 1 g/l and 10^6 autoclaved spores/ml of *B. cinerea*; medium 3 (M3), TGY with glucose at 0.1 g/l and 10^6 autoclaved spores/ml of *B. cinerea*; medium 4 (M4), glucose at 0 g/l and 10^6 autoclaved spores/ml of *B. cinerea*; and medium 5 (M5) glucose was substituted for casein at 0.2%. M3-16 bacterial cells (100 µl) from a 48 h fresh culture on Tryptic Soy broth (Difco, USA) were inoculated in media M1 to M5, and incubated at 37 °C for 3 days on a rotary shaker (150 rpm). After centrifugation at 12000 rpm for 10 min, the supernatant from each medium culture were collected for measurement of protease activity with three independent replications for each one.

Bacterial growth and protease production. For the production and characterization of protease, *B. pumilus* M3-16 strain was grown in a 250 ml liquid medium (M6) containing 1 g/l glucose, 7.5 g/l yeast extract, 5 g/l $MgSO_4$, 5 g/l KH_2PO_4, 0.1 g/l $FeSO_4$ and 5 g/l casein as described by Olajuyigbe and Ajele (2005).

Effect of salinity on bacterial growth and protease activity. Effect of salinity on M3-16 growth was evaluated by measuring optical density at 600 nm using casein medium (M6) supplemented with a gradient of salt (0, 5, 10, 15, 20, 25 and 30% NaCl, w/v) at 37 °C for 24 h. Protease activity in the supernatant was measured as detailed in the next paragraph.

Protease assay. Protease activity was determined by incubating 500 µl of 0.5% Azocasein in Tris-HCl buffer with 100 µl of cell-free supernatant (enzyme solution) for 1 h at 37 °C. The reaction was stopped by adding 500 µl of 15% TCA (trichroloroacetic acid) and shaking. This was left for 15 min and centrifuged at 7000 rpm at 4 °C for 10 min. One ml of supernatant was added to 1 ml of 1 M NaOH and absorption was measured at 440 nm. One unit (U) of protease activity was defined as the amount of enzyme that liberated 1 micromole of tyrosine in one min at 37 °C (Olajuyigbe and Ajele, 2005).

Enzyme characterization.

Optimal temperature for activity and stability of the protease. Optimal temperature was determined by incubating the reaction mixture, as described in the previous paragraph, at different temperatures ranging from 25 to 100 °C. Thermal stability was examined by preincubation of the enzyme solution for 30 min at various temperatures (30 to 100 °C) in Tris-HCl buffer (25 mmol/l; pH 8) as described by Smaali *et al*. (2003).

pH activity and stability. The optimal pH for protease activity was measured at different pH values. The buffers used were as follows (25 mmol/l): phosphate buffer (pH 5-6), Tris-HCl (pH 7-8), borate-NaOH buffer (pH 9-10), and Na_2HPO_4-NaOH buffer (pH 11-12). Protease was assayed at 37 °C. The residual activity of the enzyme was examined by incubating the enzyme solution in the above buffers for 24 h at 37 °C before adding 500 µl of 0.5% Azocasein and the remaining activities (%) were subsequently determined.

Crude enzyme preparation. The protease producing strain M3-16 was grown in 1000 ml flask containing 400 ml of casein medium (M6) supplemented with 10% (w/v) NaCl, at 37 °C for 48 h with stirring at 160 rev min^{-1}. The culture fluid was centrifuged at 8000 rpm at 4 °C for 10 min. The supernatant was subjected to precipitation with ammonium sulphate to 80% saturation at 4 °C with constant stirring overnight. The precipitate was collected by centrifugation at 9000 rpm at 4 °C for 30 min, dissolved in an appropriate volume of 25 $mmol^{-1}$ buffer phosphate (pH 7), and extensively dialysed against the same buffer. The resultant dialysate was protease crude extract, sterilized by filtration through a 0.45 µm pore size filter and stored at -20 °C until further use for electrophoresis.

Polyacrylamide gel electrophoresis and zymogram analysis. Native PAGE was performed at 4 °C with a 10% polyacrylamide gel according to the method of Laemmli (1970) by using BioRad Mini Protean II apparatus at 100 volt. The gel was stained with Coomassie blue (R250). The SDS-PAGE was performed with 12% polyacrylamide gel. After electrophoresis, one part of the gel was stained with Coomassie blue and the other one (Native and SDS-PAGE) was incubated for 2 h into 0.2% azocasein in 25 mM Tris-HCl buffer (pH 8), and was rinsed with demineralized water. Finally, the gel was stained with Coomassie blue.

Only for SDS, after migration the gel was transferred into renaturing buffer (50 mM Tris HCl, pH 8, 2.5% Triton X100) to remove SDS. The molecular mass of the subunits was estimated with standard markers (BioRad Rang Protein Molecular Weight Markers).

PCR amplification of protease gene. Genomic DNA of *B. pumilus* strain M3-16 was extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) as previously described (Essghaier *et al*., 2009) and stored in TE buffer at -20 °C for further study and analysis. The genomic DNA was used as a template to amplify a fragment protease encoding gene by polymerase chain reaction with a pair of degenerate oligonucleotide primers F16 and R16 (Table 1). The amplified fragment obtained (product I) was purified with a QIAEX gel elution kit (Qiagen) and cloned using the Gene Jet Kit Cloning (Fermentas) and transformed into INVαF' competent cells (Invitrogen). The positive recombinant clone was screened and individually cultured in LB broth supplemented with 50 µg/ml ampicillin on a rotary shaker at 37 °C for 20 h and harvested by centrifugation (8000 *x g*, 10 min). The extraction and purification of plasmids were obtained by using the Gene Jet TM Plasmid Miniprep Kit (Fermentas) and eluted in distilled water. DNA sequencing and analysis were performed by an automated system (GATC Biotech, Germany). After analysis by BLAST program; the sequence obtained was used as an original sequence for TAIL-PCR.

TAIL-PCR. In the efficient method thermal, asymmetric interlaced (TAIL) PCR was used to extend a DNA fragment adjacent to known sequence of protease (original sequence) with 489 bp as previously described (Liu and Huang, 1998). The TAIL-PCR helped to develop the DNA sequence directly from Genomic DNA using three Nested-PCR; specific primer designed from the original sequence (fragment I) in consecutive reactions together with degenerate primers. To determine flanking sequences of product I, a three-step PCR reaction technique can be applied using spe-

TABLE 1 - Oligonucleotides primers used for PCR

Name	Sequence primer	Note	Origin
F16	5′-GAY GGN GTN GAY CTN GAY TGG-3′	Degenerate primer	This study
R16	5′-RTT RTA YAA RAA NGG NAC YTT-3′	Degenerate primer	This study
F1	5′-CAG GAC CTC CTG GAG TAG GGA AA-3′	Specific primer	This study
F2	5′-TCG CGG GCA TAG AAG AAC GTA T-3′	Specific primer	This study
F3	5′-CCA CTG CCA ACA ATT TAG CAA C-3′	Specific primer	This study
R1	5′-TTG GTT GCT AAA TTG TTG GCA GTG-3′	Specific primer	This study
R2	5′-CTT CAA GCA TTG CAG AGG ATG G-3′	Specific primer	This study
R3	5′-GCA CAC GTT CTT TGA CCT TTT CCA-3′	Specific primer	This study
AD1	5′-NTCGASTWTSGWGGT-3′	Degenerate primer	Liu and Huang, 1998
AD2	5′-NGTCGASWGANAWGAA-3′	Degenerate primer	Liu and Huang, 1998
AD3	5′-WGTGNAGWANCANAGA-3′	Degenerate primer	Liu and Huang, 1998
AD4	5′-AGWGNAGWANCAWAGG-3′	Degenerate primer	Liu and Huang, 1998

cific primers (F1, F2, F3, R1, R2 and R3) synthesized based on the same sequence (fragment I) as well as a degenerate primers AD1, AD2, AD3 and AD4 (Table 1). All PCR reactions were performed using the PCR protocol with 42 cycles of amplification and primer annealing at 62 °C. In this study, 3 phases of TAIL-PCR were carried out using the same procedure described by Liu and Huang (1998).

Sequence analysis. The PCR products screened were purified, cloned, transformed and sequenced. The sequence data, both DNA and deduced amino acid sequence, were analyzed using the BLAST server (http://www.ncbi.nlm.nig.gov/blast).

RESULTS AND DISCUSSION

In our recent previous works (Sadfi-Zouaoui *et al*., 2008; Essghaier *et al*., 2009), several antagonistic halophilic bacteria were selected for their biocontrol activities and abilities to produce some antifungal enzymes (cellulase, protease, chitinase and glucanase). In the present study, we investigate the proteolytic activity of the halotolerant bacterium *Bacillus pumilus* strain M3-16.

Production and induction of protease from *Bacillus pumilus* M3-16

The induction of protease was tested by two carbon sources and/or presence of *B. cinerea* autoclaved spores using different media M1, M2, M3, M4 and M5. The results presented in Fig. 1, showed that glucose used as a sole carbon source at 1 g/l in media M1 and M2, either in presence or absence of the pathogen, inhibited the protease activity (Fig. 1). The highest production of protease by strain M3-16 (27.4 U/ml) was found in medium (M3) containing a low concentration of glucose (0.1 g/l) plus pathogen spores. This activity can also be induced by casein (0.2%) as a sole carbon source in medium M5 (3.35 U/ml).

These results showed that the production of protease is dependent on the type of carbon source used and its concentration in the medium besides the presence or absence of autoclaved spores of the pathogen. Similar data were shown in the comparative study of effect of glucose concentration on protease production from *Bacillus* sp. P02 by Patel *et al*. (2005), who found large repression of protease production by the presence of glucose at above 0.5% (w/v). From our knowledge, we firstly report a protease activity from *B. pumilus* induced by autoclaved spores of *B. cinerea*. This characteristic distinguishes it from other proteases showed until now (Patel *et al*., 2005). Several authors reported the antifungal potential of protease enzymes (Schisler *et al*., 2004; Siadiqui *et al*., 2005; Tian *et al*., 2006). These results clearly suggested the prominent role in biocontrol domain by applying protease from bacteria group against plant pathogens, pest and nematodes (Tian *et al*., 2006).

Effect of salt on growth and protease activity of strain M3-16

Protease production was maximal (3.53 U/ml) at 30% (w/v) salt followed by 3.49 U/ml at 10%, 3.44 U/ml at 25% (w/v) salt. In the absence of salt, enzyme production was also detected at the same level as observed with 10 and 30% salt (Fig. 2). The ability of the moderately halophilic bacteria to grow over a very wide range of salinities make them very attractive for biotechnological applications (Adams and Kelly, 1995).

For this study, we selected the extremely halotolerant proteolytic bacterium identified as *B. pumilus*, strain M3-16. Its protease enzyme was significantly produced in the absence and at high salt concentration 10-30% (w/v) which clearly indicated the high salt tolerant nature of the enzyme. By comparison with few studies on halo-tolerance of protease enzyme, these characteristics distinguish it from other proteases produced from halophilic bacteria, these latter cannot produce protease in the absence of NaCl (Patel e*t al.,* 2005). These results could be compared with *B. subtilis* FP-133 from which protease secretion was optimal at 5% (Setyorini *et al.,* 2006); a much higher salt requirement (25%, w/v) for a protease secretion was reported in *Filobacillus* sp. RF2-5 (Hiraga *et al*., 2005). Proteases with these characteristics may have interesting applications in biotechnological processes, such as treatment of saline waters or waste solutions with protein residues and high salt contents. In addition, for the production of stable salt tolerant enzyme from extrêmophiles, it is important to use halophilic microorganisms as producers (Coronado *et al*., 2000).

Protease characterization

Optimum temperature for the protease of *B. pumilus* M3-16 was of 60 °C (Fig. 3A), and the enzyme maintained stability (more than 91% of the original activity) in the range of temperature from 25 to 80 °C. Figure 3B shows that pH 8 was the

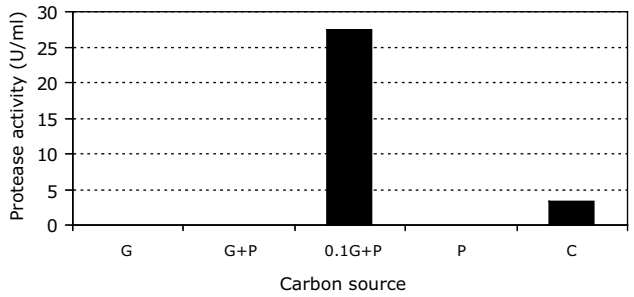

FIG. 1 - Effect of carbon source and pathogen on protease production by *Bacillus pumilus* M3-16. Protease activities were determined in cell-free supernatant of M3-16 strain tested in different culture media. G: 1 g/l glucose (M1); G+P: glucose at 1 g/l and autoclaved spores of *Botrytis cinerea* (10^6 spores/ml) (M2); 0.1G+P: 0.1 g/l glucose and autoclaved spores of *B. cinerea* (10^6 spores/ml) (M3); P: autoclaved spores of *B. cinerea* (10^6 spores/ml) (M4), and C: 0.2% casein (M5).

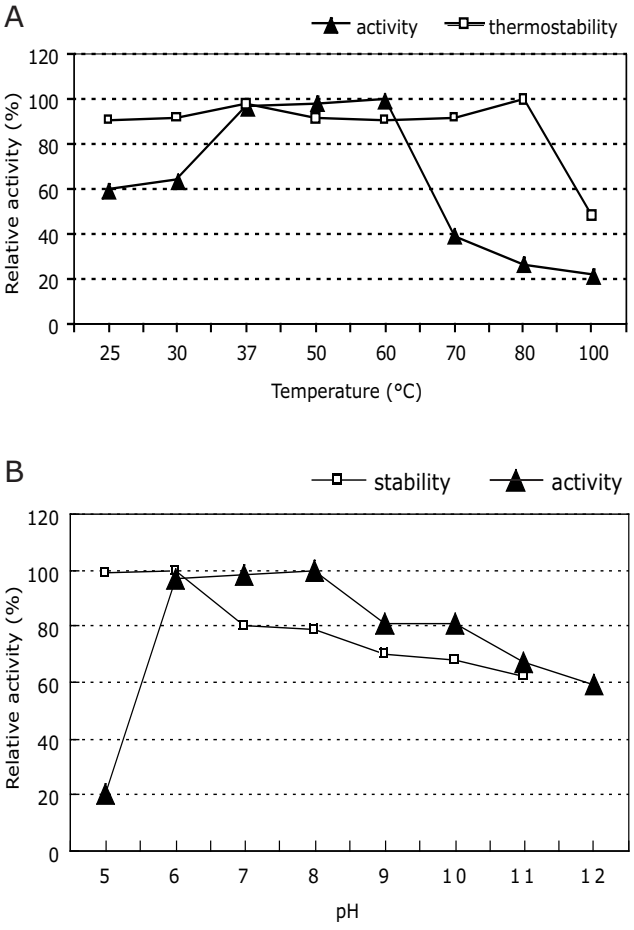

FIG. 3 - Effect of temperature and pH on protease activity of *Bacillus pumilus* M3-16. A: Effect of temperature on activity and stability of the protease enzyme at pH 8. B: Effect of pH on activity and stability of protease at 37 °C.
Relative activity is expressed as a percentage of the maximum activity (100%) under conditions used

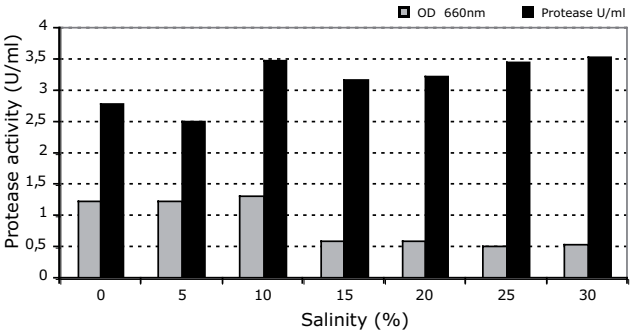

FIG. 2 - Effect of salinity on growth and protease activity of *Bacillus pumilus* M3-16 tested by incubation at 37 °C for 24 h, in presence of NaCl concentrations at 0, 5, 10, 15, 20, 25 and 30% (w/v). Influence of salt concentration on growth was measured at 660 nm and protease activity present in cell-free supernatant at 440 nm.

optimum at 37 °C, and more than 62% of protease activity was retained at pH values from 5 to 11. Thus, protease from *B. pumilus* strain M3-16 can be classified as an alkaline protease. The optimum pH 8 for protease is common among many *Bacillus* species (Ghorbel *et al.,* 2003; Nascimento and Martins, 2004; Olayjuyigbe and Ajele, 2005).

The optimum temperature of 60 °C recorded for *B. pumilus* M3-16 protease was also similar to many reported proteases from *Bacillus* species (Nascimento and Martins, 2004; Wang *et al.,* 2006) and with the values reported for few thermophiles (Dhandapani and Vijiayragavan, 1994; Kaur *et al.,* 1998; Banerjee *et al.,* 1999). It is notable that halophilic microorganisms produce thermostable alkaline proteases depending on the environmental conditions and the survival statues of the microorganisms. These agree with the results of Kumar (2002) on thermostable protease produced by alkalophilic bacteria. Further, the observed thermostability and activity values of proteases suited well with the most of the industrial processes in which enzymes employed function at temperatures exceeding 45 °C.

After electrophoresis in polyacrylamide SDS-gel, the protease was observed as a single Coomassie stained band indicating that it is a monomer protein with a relative molecular mass of 31 kDa on SDS-PAGE. In literature, the alkaline protease, from *Bacillus* sp. is reported as a single band with molecular weight ranging from 16 to 32 kDa (Adinarayana *et al.*, 2004). It should be noted that, the serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine protease Rao *et al*. (1998).

Identification and characterization of sequence protease gene

Starting by the fragment I (489 bp), we successfully obtained a fragment of 1053 bp by using a TAIL-PCR method (Fig. 4). The protease gene sequence of *B. pumilus* strain M3-16 have been deposited in the GenBank database, under the accession number FJ595839. The BLAST analysis of the nucleotide sequence obtained (1053 pb) showed a high homology (90%) with the sequence of *B. pumilus* SAFR-032, coding to class III heat-shock ATP-dependent Lon protease. The analysis of the deduced amino-acid sequence obtained (345 aa) showed a high homology of 94 and 93% respectively with the ATP-dependent protease La from *B. Pumilus* ATCC7061 and the class III heat-shock ATP-dependent Lon protease from *B. pumilus* SAFR-032. This result was expected since in literature, serine protease group was largely distributed in *Bacillus* genus.

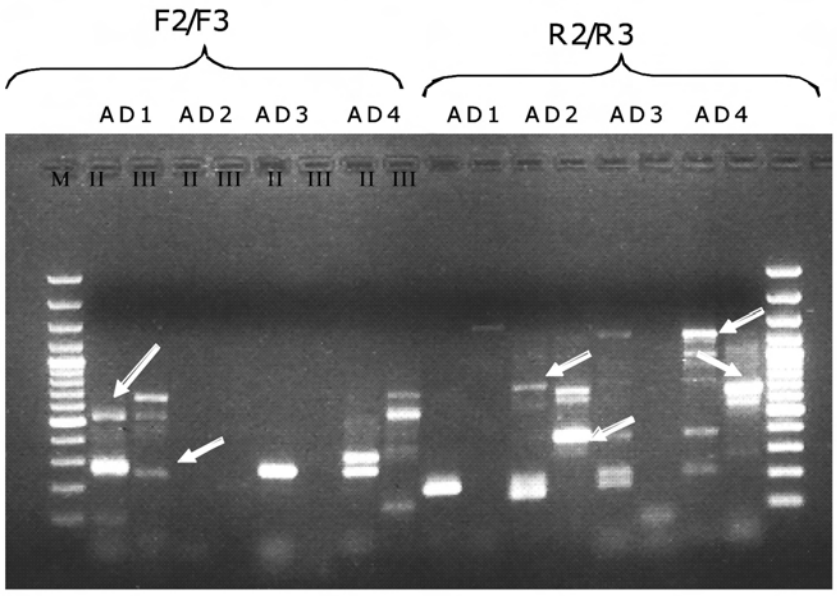

FIG. 4 - Agarose gel analysis of TAIL-PCR products to determine the downstream regions of fragment I. Ten microlitres of PCR products from each primer. We observe PCR products from second and third stapes amplification (II and III). Primers F2/F3 used for extension of region 3′ and (R2/R3) for extension of extremity 5′ of original sequence firstly obtained by ordinary-PCR. AD1, AD2, AD3 and AD4 were arbitrary degenerate primers. Arrows indicate fragments that were screened and cloned for subsequent DNA sequencing (preliminary TAIL-PCR extended 5′ and 3′ regions). Lane M, 100-bp DNA ladder (Promega).

CONCLUSION

This study showed that *B. pumilus* strain M3-16 produced very stable protease in pH alkaline, at high temperature and at high salt concentrations and inducible by the presence of the pathogen *in vitro*. Therefore, the biotechnological exploitation of this enzyme could be of great importance in agro-industries.

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