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Production of ChiA-Pt70, a new organic solvent tolerant extracellular chitinase from *Paenibacillus timonensis* strain LK-DZ15

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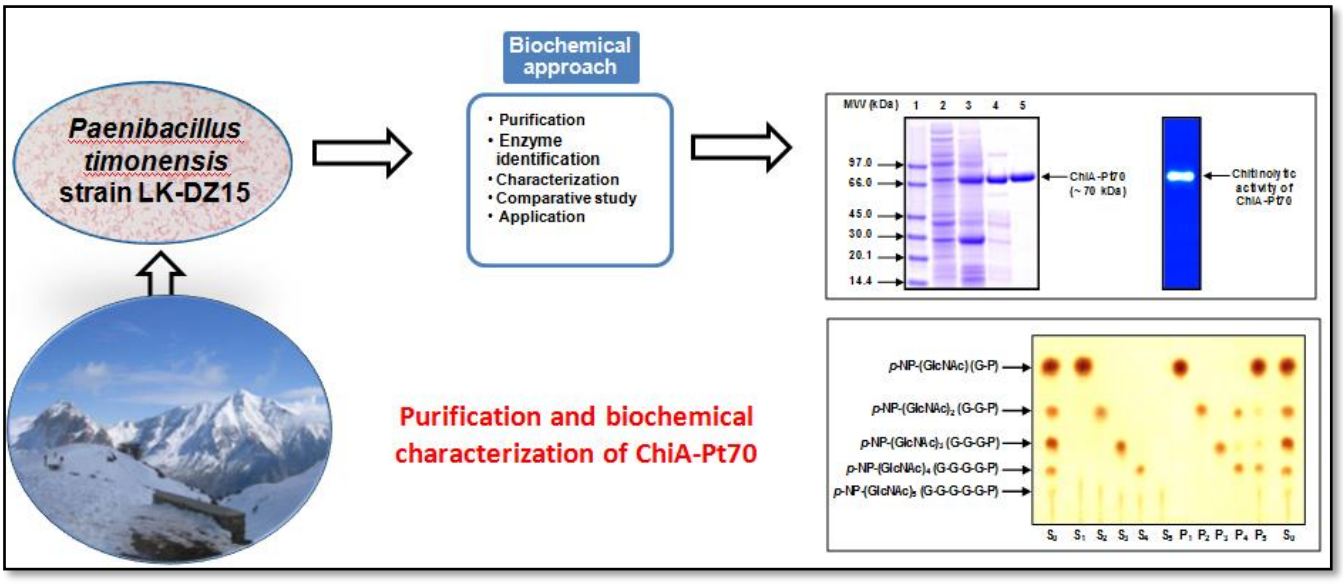
Graphical Abstract



Abstract

A new extracellular chitinase (ChiA-Pt70) was produced and purified from a newly isolated *Paenibacillus timonensis* strain LK-DZ15. The maximum chitinase activity recorded after 44-h of incubation at 30°C was 11,500 U/mL. Pure enzyme was obtained after ammonium sulphate precipitation (40-70%) followed by sequential column chromatographies on fast performance liquid chromatography (FPLC) and high performance liquid chromatography (HPLC). Based on matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) analysis, the purified enzyme is a monomer with a molecular mass of 70,166.11 kDa. The sequence of the 25 NH_2-terminal residues of the mature ChiA-70 showed high homology with *Paenibacillus* GH-18 chitinases family. Optimal activity was achieved at pH 4.5 and 80°C. The pure enzyme was completely inhibited by *p*-chloromercuribenzoic acid (*p*-CMB) and *N*-ethylmaleimide (NEM). Chitinase activity was high on colloidal chitin, chitin azure, glycol chitin, glycol chitosane, chitotriose, and chito-oligosaccharide while it did not hydrolyse chitibiose and amylose. Furthermore, thin-layer chromatography (TLC) analysis from enzymatic catalyzed hydrolysis of chitin-oligosaccharides showed that ChiA-Pt70 acted as an *endo*-splitting enzyme. Its K_m and k_{cat} values were 0.611 mg colloidal chitin/mL and 87,800 s^{-1}, respectively. Interestingly, its catalytic efficiency was higher than those of chitinases ChiA-Mt45 from *Melghiribacillus thermohalophilus* strain Nari2A^T, ChiA-Hh59 from *Hydrogenophilus hirchii* strain KB-DZ44, Chitodextrinase® from *Streptomyces griseus*, and *N*-acetyl-β-glucosaminidase® from *Trichoderma viride*. Therefore, ChiA-Pt70 exhibited remarkable biochemical properties suggesting that it is suitable for the enzymatic degradation of chitin.

1. Introduction

Chitinases are essential glycoside hydrolases (GHs) that catalyze the hydrolysis of β-1,4-glycosidic bonds of chitin in glycoconjugates, oligo- and polysaccharides. The *endo*-chitinases cleave randomly at internal sites of chitin, generating soluble low mass multimers of GlcNAc such as chitotetraose, chitotriose, and chitobiose [1, 2]. Several chitinases have been isolated and characterized from various sources [3-5, 43]. They are widely found in nature, occurring in fungi, bacteria, viruses, insects, animals and plants. They exercise various functions, such us defense, nutrient digestion, pathogenesis, and morphogenesis [5]. One of the applications of chitinases is for the bioconversion of chitin wastes from food processing industry into pharmacological active products, chito-oligosaccharides and *N*-acetylglucosamine (NAG) and bioremediation. Production of chitin derivatives with suitable enzyme is more appropriate for a sustainable environment than using chemical reactions [6]. Potential roles of chitinases in bio-control of insects and mosquitoes and in production of single cell protein have also been suggested [7]. Thus, there have been many reports on cloning, expression and characterization of chitinases from various organisms, including bacteria, fungi, plant and animals [7]. In addition, chitinases are essential for the enzymatic production of $(GlcNAc)_n$ and GlcNAc, whose physiological roles are gaining increasing attention in recent research. Accordingly, research on these enzymes in various organisms should also be of use in the production of $(GlcNAc)_n$ and GlcNAc [8].

The present study aims to report on the purification and biochemical characterization of a new chitinase enzyme (ChiA-Pt70) from *Paenibacillus timonensis* strain LK-DZ15, newly isolated from a soil sample collected from the Djurdjura Mountains in Kabylia, Algeria. The characterization of its biochemical properties suggests that this chitinase is appropriate for various industrial applications, including bioconversion of colloidal chitin into *N*-acetyl glucosamine and chitobiose.

2. Materials and Methods

2.1. ChiA-Pt70 purification procedure

Five hundred mL of a 44 h culture of *Paenibacillus timonensis* strain LK-DZ15 was centrifuged for 30 min at 9,000*g* to remove microbial cells. The supernatant containing extracellular chitinase was used as the crude enzyme preparation and was submitted to the following purification steps. The supernatant was precipitated between 40 and 70% ammonium sulfate saturation. The precipitate was then recovered by centrifugation at 9,000*g* for 30 min, resuspended in a minimal volume of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing 2 mM $MgCl_2$ at pH 6.2 (Buffer B), and dialyzed overnight against repeated changes of buffer B. Insoluble material was removed by centrifugation at 9,000*g* for 30 min. The obtained sample was subjected to chromatography purification. The supernatant was loaded on a FPLC system using an UNO Q-12 column (Bio-Rad Laboratories, Inc., Hercules, CA, USA) equilibrated in Buffer B. The column (15 mm × 68 mm) was rinsed with 500 mL of the same buffer. Adsorbed material was eluted with a linear NaCl gradient (0–500 mM in buffer B) at a rate of 60 mL/h. After being washed with the same buffer B, the unabsorbed protein fractions were eluted. Fractions showing chitinase activity were pooled and applied to a HPLC system using a Zorbax PSM 300 HPSEC (26.2 mm × 250 mm), Agilent Laboratories, pre-equilibrated with 25 mM Tricine buffer at pH 7.9 supplemented with 2 mM $CaCl_2$ (Buffer C). Proteins were separated by isocratic elution at a flow rate of 45 mL/h with buffer C and detected using a UV–Vis Spectrophotometric detector (Knauer, Berlin, Germany) at 280 nm. The pooled fractions, with retention time (R_t) of 8.979 min and containing chitinase activity, were concentrated in centrifugal micro-concentrators (Amicon Inc., Beverly, MA, USA) with 30-kDa cut-off membranes and were stored at -20°C in a 20% glycerol (v/v) solution and then used for further analysis.

2.2. Protein quantification, electrophoresis, and mass spectrometry

Total protein contents were determined according to the method of Bradford (1976) using BSA as a standard [15]. 12% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970) using a 5% stacking gel and 10% resolving gel under reducing conditions [16]. The molecular mass estimated for the native and purified chitinase was determined by PAGE under denaturating and non-denaturating conditions. The protein bands were visualized with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) staining. Zymography analysis was monitored as reported by Laribi-Habchi et al. [14, 17] using chitin azure as substrate with slight modification. Discontinuous substrate SDS-PAGE (Zymogram analysis) was performed with a 4% stacking gel, except that 1 mg/ml of heat-denatured chitin azure was incorporated into the 12% separation gel. Electrophoresis was performed at a constant current of 30 mA. After electrophoresis, the gel was immersed with 100 mL of refolding buffer (Buffer A, 2% Triton X-100) overnight at 80°C to replace the SDS and separation buffer in the gel. The gel was washed with distilled water and then stained with 0.01% (w/v) calcofluor white M2R in 50 mM Tris-HCl (pH 8). After 5 min, the brightener solution was removed and the gel was washed with distilled water. Lytic zones were visualized by placing the gels on a UV-transilluminator [18]. The molecular mass of purified ChiA-Pt70 was analyzed in linear mode by MALDI–TOF/MS using a Voyager DE-RP instrument (Applied Biosystems/PerSeptive Biosystems, Inc., Framingham, MA, USA). Data were collected with a Tektronix TDS 520 numeric oscillograph and analyzed using the GRAMS/386 software (Galactic Industries Corporation, Salem, NH, USA).

2.3. Edman degradation

Bands of purified ChiA-Pt70 were separated on SDS gels and transferred to a ProBlott membrane (Applied Biosystems, Foster City, CA, USA), and the NH_2-terminal sequence analysis was performed

by automated Edman's degradation, using an Applied Biosystem Model 473A gas-phase sequencer (Perkin–Elmer, Applied Biosystems Division), in the liquid-pulse mode. Residues of amino-acids were detected as individual signals. The sequence was compared to those in the Swiss-Prot/TrEMBL database by BLAST homology search (www.ncbi.nlm.nih.gov/blast).

2.4. Biochemical characterization of the purified chitinase ChiA-Pt70

2.4.1. Influence of metallic ions, specific inhibitors, and reducing agents

Chemical reagents, *p*-chloromercuribenzoic acid (*p*-CMB), 5,5'-dithio-bis-2-nitro benzoic acid (DTNB), NEM, iodoacetamide (IAM), iodoacetic acid (IAA), LD-dithiothreitol (LD-DTT), 2-mercaptoethanol (2-ME), 2,4,6-trinitrobenzenesulfonic acid (TNBS), phenylmethylsulfonyl fluoride (PMSF), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), diethylpyrocarbonate (DEP), *N*-bromosuccinimide (NBS), and *N*-acetylimidazole (NAI), were investigated at various concentrations for their effects on enzyme activity. Chitinase activity measured in the absence of any inhibitor or reducing agent was taken as control (100%). The effects of different divalent (Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Ba^{2+}, Fe^{2+}, Ag^{2+}, Al^{2+}, Cd^{2+}, Hg^{2+}, and Ni^{2+}) metallic ions, at 2 and 10 mM, on chitinase activity were investigated by adding them to the reaction mixture. The non-treated and dialyzed enzyme was considered as 100% for metallic ion assay.

2.4.2. Determination of pH on chitinase activity and stability

Enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on charge distribution of both substrate and particularly enzyme molecules [19-21]. The effect of pH on chitinase activity was assessed over the range of pH 2–10 under standard assay conditions. The following buffer systems, supplemented with 2 mM $CaCl_2$, were used at 50 mM: glycine-HCl for pH 2–3, citrate for pH 3–5, MES for pH 5–6, HEPES for pH 6–8, Tris-HCl for pH 8–9, and glycine-NaOH for pH 9–10. The pH stability was tested by pre-incubation of the purified chitinase in buffers with different pH from 2 to pH 6 at standard assay temperature for 12 h.

2.4.3. Determination of temperature on chitinase activity and stability

To study the effect of temperature on chitinase activity, a standard assay was performed at temperatures ranging from 40 to 100°C at intervals of 5°C. Enzyme thermostability was assessed by incubating chitinase at 70, 80, and 90°C without any substrate for 12 h in the presence and absence of 2 mM $CaCl_2$, after which residual enzyme activity was measured using the standard assay. The non-heated enzyme was used as control (100%).

3. Results and discussion

3.1. Enzyme purification

The supernatant was obtained by the centrifugation of a 44-h old culture, of the *Paenibacillus timonensis* strain LK-DZ15 using broth (500 mL) as a crude enzyme solution according to the procedure described in Section 2. Briefly, the pure ChiA-Pt70 enzyme was obtained after ammonium sulfate precipitation (40–70%) followed by sequential column chromatographies on FPLC and HPLC. Fractions corresponding to chitinase activity were pooled, and then loaded on a UNO Q-12 column using FPLC system. The protein elution profile obtained at this purification step indicated that the chitinase eluted at 120–190 mM NaCl. Purification to homogeneity was achieved using a Zorbax PSM 300 HPSEC using HPLC system. The fraction containing the chitinase activity was eluted at R_t of 8.979 min.

3.2. Molecular weights determination and zymography analysis of ChiA-Pt70

The homogeneity of the purified enzyme was also checked by SDS-PAGE under reducing conditions and by protein staining analysis. A unique protein band was obtained for the purified enzyme. The purified ChiA-Pt70 enzyme had a molecular weight of approximately 70 kDa (Fig. 1) and clear chitinase activity (Fig. 1). MALDI–TOF/MS analysis confirmed that the purified ChiA-Pt70 had an exact molecular mass of 70,166.11 kDa. These results suggested that ChiA-Pt70 was a monomeric protein comparable to those previously reported for other bacterial chitinases [13, 14, 23-29, 43]. Considerable variation in the molecular weight of chitinase had been reported earlier *viz*. 65 kDa for ChiA-65 from *Bacillus licheniformis* strain LHH100 [14], 62 kDa for Chit62 from *Serratia marcescens* strain B4A [30], 60 kDa for *Moritella marina* strain ATCC 15381^T [31], 59 kDa for ChiA-Hh59 produced by *Hydrogenophilus hirschii* strain KB-DZ44 [13], 55 kDa for ChiA from *Sanguibacter antarcticus* KOPRI 21702 [32], 51.66 kDa for for ChiA from *Pseudomonas* sp. strain TXG6-1 [33], 45 kDa for ChiA-Mt45 from *Melghiribacillus thermohalophilus* strain Nari2A^T [12], and 35 kDa for ChiA from *Paenibacillus pasadenensis* strain NCIM 5434 [11]. Generally, the molecular weight of chitinase was ranged from 30 to 72 kDa.

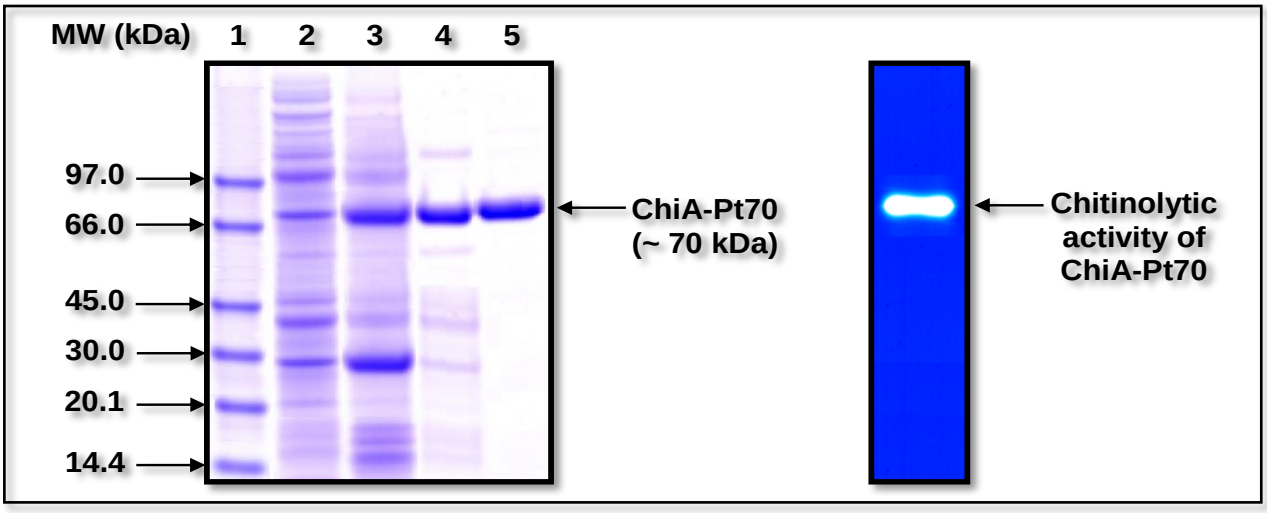


Fig.1. SDS-PAGE of the purified chitinase ChiA-Pt70. Chitin azure zymography staining of the purified chitinase ChiA-Pt70 (50 µg).

3.3. NH_2-terminal amino acid sequences of ChiA-Pt70

The twenty seven NH_2-terminal amino acid residues were determined to be AAAWAPNTSYKWYDLVSYGGSEYQCLQ. Alanine is the first NH_2-terminal amino acid residue suggesting that a signal peptide involved in secretion is cleaved from a precursor to form the mature extracellular ChiA-Pt70. The sequence showed high homology with other *Paenibacillus* chitinases, reaching 81 and 79% identity with the chitinase PtChiA71 from *Paenibacillus elgii* strain NBRC 100335 and the chitinase PeChiA68 from *Paenibacillus elgii* strain HOA73. In addition, the sequence showed homology with other *Paenibacillus* chitinases, reaching 75, 70, 59, and 57% identity with the chitinases: PeChi71 from *Paenibacillus ehimensis* strain NBRC 15659, PcChi71 from *Paenibacillus chitinolyticus* strain NBRC 15660, PaChi64 from *Paenibacillus alvei* strain DSM 29, and PdChiD from *Paenibacillus dendritiformis* strain C454, respectively. Likewise, the sequence analysis suggested that the ChiA-Pt70 enzyme is closely related with other *Bacillus* chitinases and probably evolved for a relevant short time (owing to significant similarities). Those results strongly suggested that the ChiA-Pt70 enzyme from *Paenibacillus timonensis* strain LK-DZ15 was a new member of *Paenibacillus* GH-18 chitinases family.

3.4. Biochemical characterization of the purified chitinase

3.4.1. Chemical modification and effect of metallic ions on the activity of ChiA-Pt70

The activity of ChiA-Pt70 from *Paenibacillus timonensis* strain LK-DZ15 was found to be totally inhibited in the presence of *p*-CMB, DTNB, and NEM. Partial activity loss was observed when it was incubated with IAM, IAA, LD-DTT, and 2-ME. This indicates the presence of sulfhydryl groups on active site of the enzyme, as confirmed by total inhibition observed in the presence of mercuric ionThe chitinases from *Melghiribacillus thermohalophilus* strain Nari2A^T [12], *Bacillus licheniformis* strain LHH100 [14], *Hydrogenophilus hirschii* strain KB-DZ44 [13], and *Streptomyces* sp. strain M-20 [34] were completely inhibited by *p*-CMB. EDC did not inhibit the activity of the enzyme, suggesting that the glutamic acid residue in the active site was not accessible to EDC. This behavior was similar to that observed for the purified chitinases ChiA-Mt45 from *Melghiribacillus thermohalophilus* strain Nari2A^T [12], ChiA-65 from *Bacillus licheniformis* strain LHH100 [14], and ChiA-Hh59 from *Hydrogenophilus hirschii* strain KB-DZ44 [13].
The obtained results showed that the test with 2 mM was retained since there was no difference with that of 10 mM. Among all tested metallic ions, only Cd^{2+}, Hg^{2+}, and Ni^{2+} completely inhibited enzyme activity, while Ag^{2+} and Al^{2+} reduced enzyme activity by 84, and 33%, respectively. Other reagents such as Cu^{2+} did not show significant inhibition or activation effect on the chitinase. However, the enzyme activity of chitinase was significantly increased with the addition of 2 mM of Ca^{2+}, Mn^{2+}, and Mg^{2+} where the relative activity was recorded to be 235, 175, and 140%, respectively. The increased activity in the presence of Ca^{2+} implies that this cation plays an important role in the regulation of enzyme active conformation and in this way increases chitinolytic activity. Hg^{2+} was found to be the major inhibitor of chitinase activity since it reacts with –SH groups found in cysteine residues in the protein chain and disrupts the tertiary structure [35]. It strongly inhibits chitinases from different genera [25]. Report on the effect of metallic ions on chitinases is quite divers [14, 23, 36].

3.4.2. Effects of pH on the activity and stability

The effect of pH on the catalytic activity was studied by using colloidal chitin as a substrate under the standard assay conditions. The enzyme was active in pH range from pH 2–10 with maximum activity at pH 4.5 (Fig. 2). The relative activities at pH 3 and 7 were 75 and 77%, respectively. In agreement to the current study, chitinases from *Sulfolobus tokodaii* strain 7 [37] and *Paenibacillus thermoaerophilus* strain TC22-2b [38] have maximum activity at pH 2.5 and 4, respectively. Likewise, the chitinase ChiA-65 from *Bacillus licheniformis* strain LHH100 have an optimum pH at 4 [14]. However, the chitinases of *Paenibacillus* sp. strain D1 [9], *Paenibacillus illinoisensis* strain KJA-424 [10], *Streptomyces violaceusniger* strain MTCC 3959 [39], and *Hydrogenophilus hirschii* strain KB-DZ44 [13] show the optimum pH at 5. Otherwise, the chitinases of *Paenibacillus pasadenensis* strain NCIM 5434 [11] and *Sanguibacter antarcticus* strain KOPRI 21702 [32] have an optimum pH at 10 and 7.6, respectively. Bacterial chitinases are active over a wide range of pH, depending on the source of the bacteria from which they have been isolated [19, 40].
The pH stability profile of purified ChiA-Pt70 indicated that this enzyme was highly stable in the pH range of 2–6 (Fig. 2). The half-life times of ChiA-Pt70 at pH 2, 3, 4, 5, and 6 were 9, 7, 4, 2, and 1 h, respectively. The wide range of pH stability of ChiA-Pt70 will be very useful for industrial and commercial applications performed at acidic conditions.

3.4.3. Effects of temperature on the activity and stability

The optimum temperature recorded for the activity of the purified chitinase ChiA-Pt70 at pH 4.5 was 70°C in the absence of $CaCl_2$ and 80°C in the presence of 2 mM Ca^{2+}, using colloidal chitin as substrate (Fig. 3). The optimal temperature of ChiA-Pt70 was found to be much higher than those of most other reported bacterial chitinases with optimal temperatures in the range of 28-60°C [11, 31, 36]

and 75-80°C [14]. While the chitinase from *Bacillus thuringiensis* subsp. *kurstaki* strain HBK-51 showed maximum activity at 110°C [41].

The half-life times at 70, 80, and 90°C in the absence of Ca^{2+} were 8, 5, and 2 h, respectively. However, in the presence of 2 mM Ca^{2+}, the half-life times of ChiA-Pt70 increased to 10, 6, and 3 h. In fact, the Ca^{2+} ion is known to play a major role in enzyme stabilization by increasing the activity and thermal stability [14, 42]. The thermostability of ChiA-Pt70 was higher than several other previously reported chitinases [14, 23, 30, 36].

The high temperature optimum and the thermal stability of the chitinase ChiA-Pt70 is particularly advantageous for its applicability to the recycling of chitin wastes. Generally, the temperature increases during bioconversion of wastes, and as the chitinase reported here has a high temperature optimum; it could be very useful at this stage of recycling.

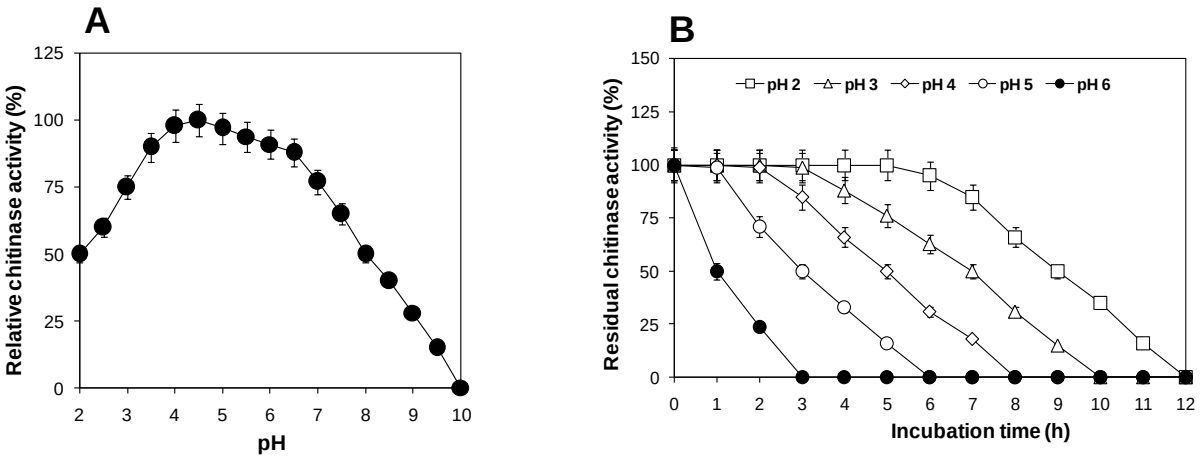


Fig. 2. Effects of pH on the activity **(A)** and stability **(B)** of the purified chitinase ChiA-Pt70 from *Paenibacillus timonensis* strain LK-DZ15. The activity of the enzyme at pH 4.5 was taken as 100%.

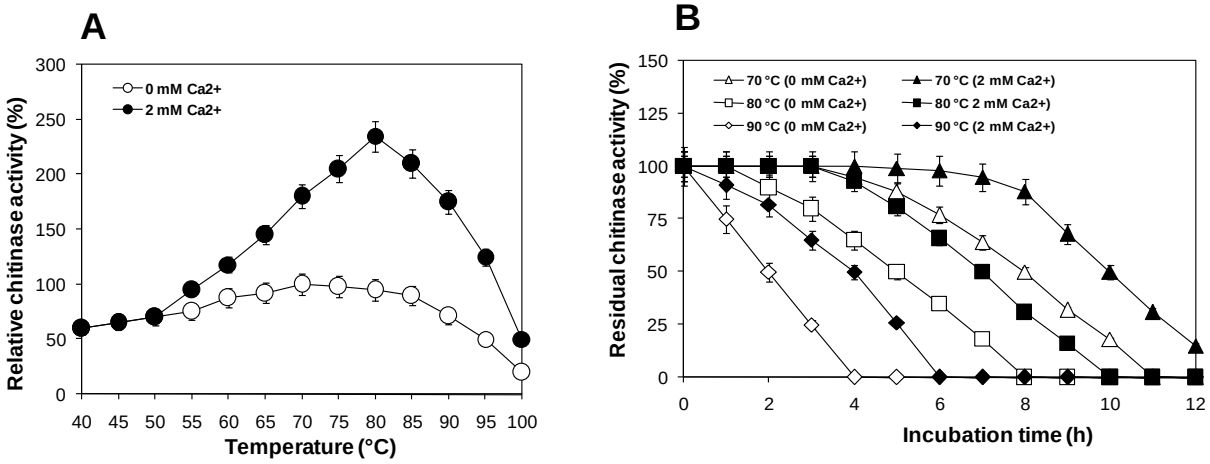


Fig. 3. Effects of the thermoactivity **(A)** and the thermostability **(B)** of chitinase ChiA-Pt70. The enzyme was pre-incubated in the absence or presence of $CaCl_2$ at various temperatures ranging from 40 to 100°C. Residual chitinase activity was determined from 0 to 12 h at 1 h intervals. The activity of the non-heated enzyme was taken as 100%. Each point represents the mean of three independent experiments.

4. Conclusion

The *Paenibacillus timonensis* strain LK-DZ15 produced significantly high amounts of extracellular thermostable chitinase (named ChiA-Pt70). The latter was submitted to a battery of purification and biochemical characterization assays. ChiA-Pt70 showed optimum activity at 80°C and pH 4.5. The ChiA-Pt70 chitinase also exhibited high levels of activity and stability over a wide range of pH, temperature and salinity which responds to the industrial requirements in bioconversion of chitin waste.

5. References

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