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Purification and biochemical characterization of a new organic solvent-tolerant chitinase from *Paenibacillus timonensis* strain LK-DZ15 isolated from the Djurdjura Mountains in Kabylia, Algeria

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ABSTRACT

A new extracellular chitinase (called 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), 5,5′-dithio-bis-2-nitro benzoic acid (DTNB), and *N*-ethyl-maleimide (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 colloidal chitin 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

Chitin, a linear β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) polysaccharide, is the major structural component of fungal cell walls, insect exoskeletons, and shells of crustaceans. It is one of the most naturally abundant occurring polysaccharides and has attracted tremendous attention in the fields of agriculture, pharmacology, and biotechnology [1–3]. Most of the chitin in nature has either an α- or a β-crystalline structure, with a predominance of the α-form. Each year, a vast amount of chitin waste is released from the aquatic food industry, where crustaceans (prawn, crab, shrimp, and lobster) constitute one of the main agricultural products. This creates a serious environmental problem, because chitin is a rotting protein. This linear polymer can be hydrolysed by bases, acids or enzymes, such as lysozyme, some glucanases, and chitinases.

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Abbreviations

FPLC	fast performance liquid chromatography
HPLC	high performance liquid chromatography
MALDI-TOF/MS	matrix assisted laser desorption ionization-time of flight/mass spectrometry
p-CMB	*p*-chloromercuribenzoic acid
DTNB	5,5′-dithio-bis-2-nitro benzoic acid
NEM	*N*-ethylmaleimide
TLC	thin-layer chromatography
GlcNAc	β-1,4-linked *N*-acetyl-D-glucosamine
NAG	*N*-acetylglucosamine
3D	three-dimensional
CBD	*p*-nitrophenyl
BSA	bovine serum albumin
DNS	5-dinitrosalycilic acid
API	analytical profiling index
LB	Luria-Bertani
IPTG	isopropyl-thio-β-D-galactopyranoside
MEGA	Molecular Evolutionary Genetics Analysis
MES	2-(*N*-morpholino) ethanesulfonic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
LD-DTT	LD-dithiothreitol
β-ME	β-mercaptoethanol
TNBS	2,4,6-trinitrobenzenesulfonic acid
PMSF	phenylmethylsulfonyl fluoride
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
DEP	diethylpyrocarbonate
NBS	*N*-bromosuccinimide
NAI	*N*-acetylimidazole
EGTA	ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acitic acid.
DMSO	dimethyl sulphoxide

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 [4,5]. Several chitinases have been isolated and characterized from various sources [6–8]. 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 [8]. 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 [9]. Potential roles of chitinases in bio-control of insects and mosquitoes and in production of single cell protein have also been suggested [10]. Thus, there have been many reports on cloning, expression, and characterization of chitinases from various organisms, including bacteria, fungi, plant, and animals [10]. 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 [11].

Various chitinases have been isolated from some bacterial strains such as *Serratia marcescens* [12], *Bacillus cereus* [13], *Bacillus licheniformis* [14], and *Stenotrophomonas maltophilia* [15]. The GHs are categorized into families (18 and 19) according to their sequences in the CAZy database (www.cazy.org) [16]. Those belonging to family 18 are distributed among bacteria, plants, animals, and other organisms [16]. On the other hand, family 19, are present mainly in higher-order plants and are reported to have strong antibacterial properties [17]. Chitinases are classified into three categories *exo*-chitinases ([EC 3.2.1.29], *endo*-chitinases [EC 3.2.1.14], and *N*-acetylglucosaminidases [EC 3.2.1.52]) according to the manner in which they cleave chitin chains. *Exo*-chitinases cleave the chain from the reducing and non-reducing end to form diacetyl-chitobiose ($GlcNAc_2$). *Endo*-chitinases randomly cleave β-1,4-glycosidic bonds of chitin, whereas *N*-acetylglucosaminases hydrolyse $GlcNAc_2$ into GlcNAc or produce GlcNAc from the non-reducing end of *N*-acetyl-chito-oligosaccharides [18]. In this regard, *exo*- and *endo*-chitinases are GH18s and *N*-acetylglucosaminidases are GH20s.

Paenibacillus sp. as a genus of facultative anaerobic bacteria, are widely found in nature [19]. Several species from this genus have been found to produce chitinases such as *Paenibacillus* sp. [20–22], *Paenibacillus illinoisensis* strain KJA-424 [23], *Paenibacillus pasadenensis* strain NCIM 5434 [24], and *Paenibacillus barengoltzii* strain CAU904 [25,26]. However, to the best of the authors' knowledge, no report is available regarding production, purification, and characterization of chitinase from *Paenibacillus timonensis*. Accordingly, 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. Substrates, chemicals, reagents, and used comparative chitinases

Chitin from shrimp shells, chitin azure, glycol chitin, glycol chitosan, laminarin, *p*-nitrophenyl *N*-acetyl-chito-oligosaccharides [*p*-NP-$(GlcNAc)_n$, n = 1–5], bovine serum albumin (BSA), 5-dinitrosalycilic acid (DNS), and calcofluor white M2R were purchased from Sigma Chemical (St. Louis, MO, USA). The well-known commercial valuable Chitodextrinase® or chitinase or poly(1,4-β-[2-acetamido-2-deoxy-D-glucoside]) glycanohydrolase from *Streptomyces griseus* and *N*-acetyl-β-glucosaminidase® or chitinase from *Trichoderma viride* obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany), the chitinases ChiA-Mt45 from *Melghiribacillus thermohalophilus* strain Nari2A^T [27], ChiA-Hh59 from *Hydrogenophilus hirchii* strain KB-DZ44 [28], and ChiA-65 from *Bacillus licheniformis* strain LHH100 [29] were used for comparison. The ZORBAX PSM 300 HPSEC column was obtained from Agilent Laboratories (Agilent Technologies, Lawrence, Kansas, MO, USA). Protein marker LMW was purchased from GE Healthcare Bio-Sciences (Marlborough, MA, USA). The Amicon Ultra-4 units (30 kDa cutoff size) was obtained from Merck Millipore (Billerica, MA, USA). The UNO Q-12 column and a protein assay kit were from Bio-Rad Laboratories (Hercules, CA, USA). All of the other chemicals and reagents used were of analytical grade or the best grade commercially available, unless otherwise stated.

2.2. Preparation of colloidal chitin

Colloidal chitin was prepared according to the method of Roberts and Selitrennikoff [30] with some modifications. Briefly, 5 kg of chitin from crab shells were gradually added into 100 mL of cold concentrated HCl with gentle agitation on a magnetic stirrer at 4 °C for 24 h. The mixture was then added to 500 mL of ice-cold 96% ethanol and left for 24 h with rapid stirring at 4 °C. The precipitate was harvested by

centrifugation at 12,000 *g* for 25 min at 4 °C and washed repeatedly with sterile distilled water until the pH reached 6. The colloidal chitin was kept at 4 °C until further use. Approximately 96 g of colloidal chitin was obtained by this procedure from 5 g of chitin powder.

2.3. Isolation and cultivation of chitinase-producing microorganisms

Samples were collected from soil of Lalla Khedidja (Tamgut Aâlayen) in Tikjda (GPS coordinates: Latitude 36°27′0″ N, Longitude 4°13′60″ E), the highest summit of the Djurdjura Mountains in Kabylia (2308 m), Algeria, using 1 L sterile thermal glass bottles. Samples were stored in the laboratory at room temperature. Enrichment cultures and isolation were performed in initial medium containing (in g/L): glucose, 2; NH_4Cl, 1.5; K_2HPO_4, 1; KH_2PO_4, 1; NaCl, 10; KCl 0.1; $CaCl_2{\cdot}2H_2O$, 1; $MgCl_2{\cdot}6H_2O$, 0.25; yeast extract, 1; and biotrypcase, 2. pH was adjusted to 7. Enrichment cultures were sub-cultured several times under the same conditions. Submerged cultures were carried out in 1000 mL shake flasks with 100 mL of medium. The flasks were inoculated and incubated in an orbital shaker at 30 °C and 180 rpm for 72 h. From each sample, 100 µL aliquot was plated by spreading on initial medium plates (at least five replicates) and incubated for 12, 24, 36, 48, 60, and 72 h at 30 °C. Different colonies were selected and restreaked several times to obtain pure cultures which were stored in nutrient agar until used. All colonies were tested for chitinase activity on the chitinase detection agar (CHDA) composed of (g/L): chitin colloidal, 20; beef-extract, 5; K_2HPO_4, 1.5; KH_2PO_4, 1.5; $MgSO_4{\cdot}7H_2O$, 1; NaCl, 5; trace elements, 2% (v/v), and 20; bacteriological agar. Chitinase producer strains were determined after 2–3 days at 55 °C by visualizing the clear zone formed surrounding the colonies on the CHDA plate. For the production of chitinase in liquid medium, the isolated LK-DZ15 strain was cultured in an Erlenmeyer flasks (1000 mL) containing the optimized medium for 96 h at 30 °C in a shaker incubator (180 rpm). Bacterial growth was estimated by measuring the optical density at 600 nm and was converted to cell dry weight (g/L) based on the biomass *versus* cell dry weight standard. In this optimal condition, the maximum chitinase activity was 11,500 U/mL. Since the chitinase activity was considerably detected and measured in the initial medium with a significant yield (2500 U/mL), the optimization of the medium with the classical method "one-factor-at-a-time (OFAT)" involves changing one independent variable (such as the nutrient, temperature, pH, etc.) while fixing others at certain levels.

2.4. Identification of microorganism, DNA sequencing, and phylogenetic analysis

Analytical profiling index (API) strip tests and 16S rRNA gene sequencing (ribotyping) were carried out for the identification of the genus to which the strain belonged. API 50 CH strips (bioMérieux, SA, Marcy-l'Etoile, France) were used to investigate the physiological and biochemical characteristics of strain LK-DZ15 in accordance with the instructions of the manufacturer. Using a set of synthetic oligonucleotides homologous to broadly conserved sequences *in vitro* amplification *via* the polymerase chain reaction (PCR) followed by direct sequencing results in almost complete nucleotide determination of a gene coding for 16S rRNA. The primer sequences were chosen from the conserved regions previously reported for the bacterial 16S rRNA. Universal primers flank hypervariable regions that can provide species specific signature sequences useful for identification of bacteria, were designed from base positions 8 to 27 and 1541 to 1525, respectively, which were the conserved zones within the rRNA operon of *E. coli* [31]. The 16S rRNA gene was amplified by PCR using forward 27F (5′-AGAGTTTGA TCCTGGCTCAG-3′) and reverse 1525R (5′-AAGGAGGTGATCCAA GCC-3′) primers. The genomic DNA of strain LK-DZ15 was purified by the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and then used as a template for PCR. Amplification conditions included an initial denaturation step 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s; annealing at 60 °C for 45 s; and an extension at 72 °C for 60 s, with a final extension at 72 °C for 10 min. The amplified ~1.5 kb PCR product was cloned in the pGEM-T Easy vector (Promega, Madison, WI, USA), leading to pLK-DZ15-16S plasmid (This study). The *E. coli* DH5α [F^- *supE 44 Φ80 δlacZ ΔM15 Δ (lacZYA-argF) U169 endA1 recA1 hsdR17* (r_k^-, m_k^+) *deoR thi-1* λ^- *gyrA96 relA1*] (Invitrogen, Carlsbad, CA, USA) was used as a host strain. All recombinant clones of *E. coli* were grown in Luria-Bertani (LB) media with the addition of ampicillin, isopropyl-thio-β-D-galactopyranoside (IPTG), and X-gal for screening. DNA electrophoresis, DNA purification, restriction, ligation, and transformation were all performed according to the method previously described by Sambrook et al. [32].

The nucleotide sequences of the cloned 16S rRNA gene were determined on both strands using BigDye Terminator Cycle Sequencing Ready Reaction kits and the automated DNA sequencer ABI PRISM® 3100-Avant Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The obtained sequences were compared with sequences available in the public sequence databases and with the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/), a web-based tool for the identification of prokaryotes based on 16S rRNA gene sequences from type strains. Phylogenetic and molecular evolutionary genetic analyses were performed using the Molecular Evolutionary Genetics Analysis (MEGA) software v. 4.1. Distances and clustering were calculated using the neighbor-joining method. Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 100 re-samplings. Multiple nucleotide sequence alignment was performed using the BioEdit version 7.0.2 software program.

2.5. Standard assay of ChiA-Pt70 activity

Chitinase activity was measured colorimetrically by detecting the amount of GlcNAc released from colloidal chitin as substrate [33]. Unless otherwise stated, 1 mg/mL of enzyme concentration was mixed with 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer supplemented with 2 mM $CaCl_2$ at pH 4.5 (i.e., Buffer A) containing colloidal chitin (10 mg/mL) as substrate and incubated at 80 °C for 1 h. The mixture was boiled for 10 min, chilled, and centrifuged to remove insoluble chitin. The resulting products of reducing sugars were measured by the DNS method described by Miller [34].

Readings were compared with a standard curve prepared with a series of dilutions of GlcNAc (from 0 to 10 mg/mL). One unit of chitinase activity was defined as the amount of enzyme required to produce 1 µmoL of GlcNAc from colloidal chitin per min under the specified assay conditions.

When using *p*-NP-$(GlcNAc)_n$ (n = 1–5) as substrate, enzyme activity was measured by the method of Ohtakara [35]. Unless otherwise stated, 250 µL of a suitably diluted enzyme solution and 250 µL of 1 mg/mL *p*-NP-$(GlcNAc)_n$ were added to 250 µL of buffer A, and this was incubated at 80 °C for 30 min. After incubation, 250 µL of 200 mM sodium carbonate at pH 11 was added, and the absorbance of the *p*-nitrophenol released was measured spectrophotometerically at 420 nm. One unit of chitinase activity was defined as the amount of enzyme releasing 1 µmoL of *p*-nitrophenol per min under the specified assay conditions.

2.6. 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 sulphate 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 (i.e., 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 $CaCl_2$ (i.e., 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 (Rt) 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.

2.7. Protein quantification, electrophoresis, and mass spectrometry

Total protein contents were determined according to the method of Bradford (1976) using BSA as a standard [36]. 12% Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970) using a 5% stacking gel and 10% resolving gel under reducing conditions [37]. 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. [29,38] 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 [39]. 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 soft-ware (Galactic Industries Corporation, Salem, NH, USA).

2.8. 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).

2.9. Biochemical characterization of the purified chitinase ChiA-Pt70

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

Chemical reagents, *p*-CMB, DTNB, NEM, iodoacetamide (IAM), iodoacetic acid (IAA), LD-dithiothreitol (LD-DTT), β-mercaptoethanol (β-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.

2.9.2. 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 [40–42]. 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.

2.9.3. 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%).

2.10. Kinetic study of the purified chitinase

2.10.1. Substrate specificity of ChiA-Pt70

The substrate specificity of the purified chitinase ChiA-Pt70 was determined using natural and synthetic substrates at various concentrations under standard assay conditions. The used substrates are: colloidal chitin, chitin azure, glycol chitin, chitin, glycol chitosan, chitibiose, chitotriose, chito-oligosaccharide, xylan, amylose, carboxymethyl cellulose, cellobiose, and laminarin. The used synthetic substrates are: *p*-NP-(GlcNAc) (G-P), *p*-NP-$(GlcNAc)_2$ (G-G-P), *p*-NP-$(GlcNAc)_3$ (G-G-G-P), *p*-NP-$(GlcNAc)_4$ (G-G-G-G-P), and *p*-NP-$(GlcNAc)_5$ (G-G-G-G-G-P). The substrates were used at concentrations, and the reaction was carried out at 80 °C for up to 24 h. The amount of reducing sugar was quantified colorimetrically, as described above for the standard assay.

2.10.2. Kinetic measurements

Kinetic parameters were calculated from the initial rate activities of the purified bacterial enzymes (ChiA-Pt70, ChiA-Mt45, ChiA-Hh59, ChiA-65, Chitodextrinase®, and *N*-acetyl-β-glucosaminidase®) at a final concentration of 1.5 mg/mL using natural (colloidal chitin) and synthetic [*p*-NP-$(Glc\text{-}NAc)_3$ (G-G-G-P)] substrates at concentrations ranging from 0.10 to 50 mg/mL at 70 °C for 5 min in assay buffer A supplemented with 5% (v/v) dimethyl sulphoxide (DMSO) and 1% (v/v) Triton X-100 at pH 4. Assays were carried out in triplicate and kinetic parameters were estimated by Lineweaver–Burk plots. Kinetic constants, Michaelis–Menten constant (K_m) and maximal reaction velocity (V_{max}) values, were calculated using the Hyper 32 v.1.0. software package developed at Liverpool University (http://hompage.ntlword.com/john.easterby/hyper32.html). The value of the turnover number (k_{cat}) was calculated by the following equation:

$$k_{cat} = \frac{V_{max}}{[E]}$$

where [E] refers to the active enzyme concentration and V_{max} to the maximal velocity.

2.11. Enzymatic performance of the purified chitinase

2.11.1. Effect of organic solvents on the stability of the purified ChiA-Pt70

Organic solvents are used for solubilizing hydrophobic substrates in enzymatic reactions; thus; various organic solvents, with different Log *P* values (25%, v/v), were tested at 50 °C and with shaking at 160 strokes per min for 12 h to evaluate their effects on chitinase stability. The residual chitinolytic activities were assayed under the same conditions of each enzyme. The chitinases used were: ChiA-Pt70, ChiA-Mt45, ChiA-Hh59, ChiA-65, Chitodextrinase®, and *N*-acetyl-β-glucosaminidase®. The activity of the enzyme without any organic solvent was taken as 100%.

2.11.2. Thin-layer chromatography of hydrolysis products

Twenty five mL of the purified chitinase ChiA-Pt70 (50 μg/mL) was incubated with 100 mL of colloidal chitin (10 mg/mL) at 50 °C for 24 h. Enzyme reactions were stopped by boiling for 5 min. After centrifugation, the supernatants were concentrated by a vacuum centrifuge and spotted onto silica gel 60 F_{254} TLC aluminum sheets (Merck, Whitehouse Station, NJ, USA). TLC plates were developed with acetonitrile-ethyl acetate-2-propanol-water (17:5:11:10, v/v/v/v) and sprayed with a mixture of methanol-sulfuric acid (95:5, v/v), followed by heating at 150 °C in an oven until spots appeared as reported elsewhere [43]. *p*-NP-$(GlcNAc)_n$ (n = 1–5) were used as standards.

2.12. Statistical analysis

All determinations were performed at least three independent replicates, and the control experiment without chitinase was carried out

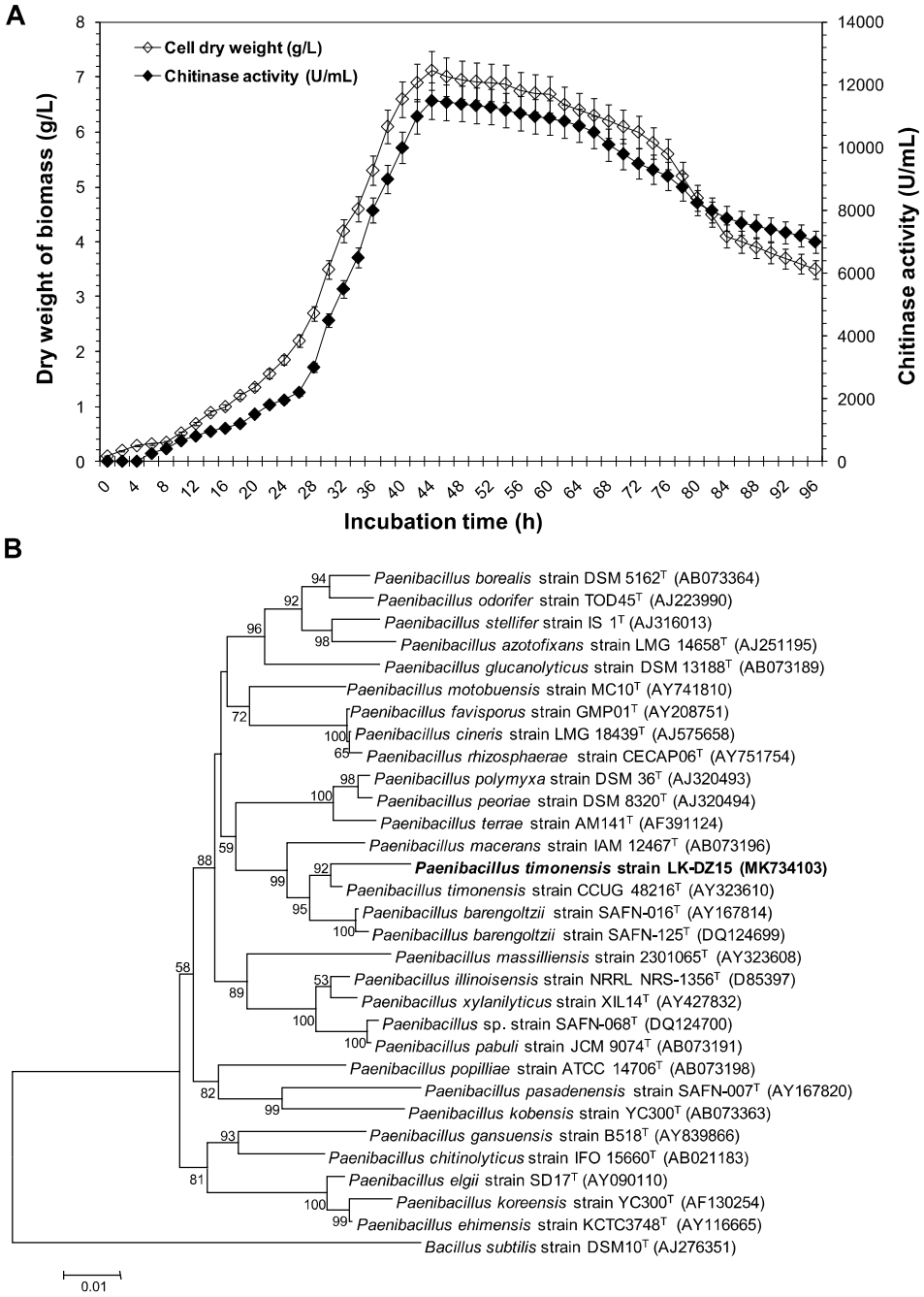

Fig. 1. (A) Time course of *Paenibacillus timonensis* strain LK-DZ15 cell growth (◇) and chitinase production (♦). Cell growth was monitored by measuring the absorbance at 600 nm and was converted to cell dry weight (g/L). Each point represents the mean of three independent experiments. **(B)** Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain LK-DZ15 (GenBank accession n°.: **MK734103**) within the radiation of the genus *Paenibacillus*. The sequence of *Bacillus subtilis* strain DSM 10T (GenBank accession n°.: AJ276351) was chosen as an outgroup. Bar, 0.01 nt substitutions per base. Numbers at nodes (> 50%) indicate support for the internal branches within the tree obtained by bootstrap analysis (percentages of 100 bootstraps). NCBI accession numbers are presented in parentheses.

under the same conditions. The experimental results were expressed as the mean of the replicate determinations and standard deviations (mean ± SD). The statistical significance was evaluated using t-tests for two-sample comparison and one-way analysis of variance (ANOVA) followed by *t*-test. The results were considered statistically significant for *P* values of less than or equal to 0.05. The statistical analysis was performed using the R package Version 3.1.1 (Vanderbilt University, USA).

2.13. Nucleotide sequence accession number

The nucleotide sequence data of 16S rRNA gene (1515 bp) reported in this paper has been submitted to the GenBank/ENA/EMBL databases under accession n°.: **MK734103.**

3. Results and discussion

3.1. Screening of chitinase-producing strains

About sixty bacterial strains that were isolated from soil samples of Lalla Khedidja (Tikjda), the highest summit of the Djurdjura Mountains in Kabylia, Algeria, and were identified as chitinase producers on the basis of their clear zone formation on CHDA plate at pH 7. The ratio of the diameter of the clear zone and that of the colony served as an index for the selection of strains with high chitinase production ability. Ten isolates were noted to exhibit a ratio that was higher than 5, with the highest ratio being 6. Strain LK-DZ15 exhibited the highest extracellular chitinase activity (about 11,500 U/mL) after 44 h incubation in an optimized medium (Fig. 1A) and was, therefore, retained for all subsequent studies.

3.2. Identification and molecular phylogeny of the microorganism

The identification of the newly isolated bacterium (designated as LK-DZ15) was based on both catabolic and molecular methods. Morphological, biochemical, and physiological characteristics, according to the methods described in Bergey's Manual of Systematic Bacteriology, showed that the LK-DZ15 isolated strain appears in a bacillus form, aerobic, endospore-forming, Gram-positive, catalase+, oxydase+, motile, and aerobic rod-shaped bacterium. Furthermore, finding from API 50 CH gallery test showed that this isolate metabolize matose, lactose, D-xylose, L-arabinose, D-tagatose, starch, galactose, and fructose besides other simple sugars. All the data obtained with regards to the physiological and biochemical properties of the isolate, therefore, strongly confirmed that the LK-DZ15 strain belonged to the *Paenibacillus* genus.

The molecular identification of the strain LK-DZ15 was realized. A 1515 bp fragment of the 16S rRNA gene was amplified from the genomic DNA of the isolate, cloned in the pGEM-T Easy vector, and sequenced on both strands. The 16S rRNA gene sequence obtained was subjected to GenBank BLAST search analyses, which yielded a strong homology with those of several cultivated strains of *Bacillus*, reaching a maximal of 99% sequence identity. The nearest *Paenibacillus* strains identified by the BLAST analysis were the *Paenibacillus timonensis* strain CCUG 48216^T (GenBank accession n°.: AY323610), *Paenibacillus barengoltzii* strain SAFN-016^T (GenBank accession n°.: AY167814), and

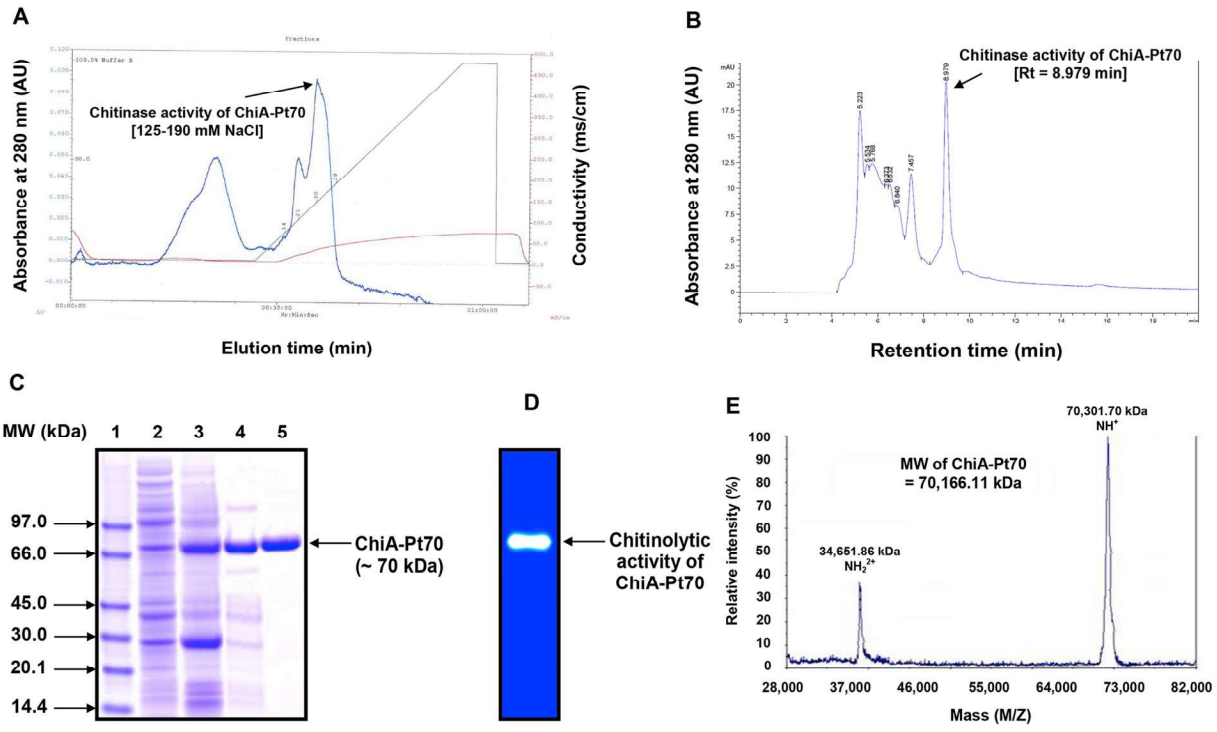

Fig. 2. (A) Chromatography of the chitinase ChiA-Pt70 from *Paenibacillus timonensis* strain LK-DZ15 on a FPLC system using UNO Q-12. The column was equilibrated with buffer A. Adsorbed material was eluted with a linear NaCl gradient (0–500 mM in buffer A) at a flow rate of 60 mL/h, and assayed for protein content at 280 nm and chitinase activity as described in Section 2. **(B)** Chromatography of the chitinase ChiA-Pt70 on HPLC system using a ZORBAX PSM 300 HPSEC. The column was equilibrated with 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. **(C)** SDS-PAGE of the purified chitinase ChiA-Pt70. Lane 1, Amersham LMW protein marker (GE Healthcare Europe GmbH, Freiburg, Germany). Lane 2, total cell extract. Lane 3, dialyzed sample after ammonium sulphate precipitation (40–70%). Lane 4, sample after FPLC (UNO Q-12) at 120–190 mM NaCl. Lane 5, purified chitinase ChiA-Pt70 (50 µg) obtained after ZORBAX PSM 300 HPLC chromatography (Rt = 8.979 min). **(D)** Chitin azure zymography staining of the purified chitinase ChiA-Pt70 (50 µg). The gel was stained with Coomassie Brilliant Blue R 250. **(E)** MALDI-TOF spectrum of 10 pmol purified chitinase ChiA-Pt70 from *Paenibacillus timonensis* strain LK-DZ15. The mass spectrum shows a series of multiple protonated molecular ions. The molecular mass of the enzyme was found to be 70,166.11 kDa. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Flow sheet for the purification of ChiA-Pt70 chitinase from *Paenibacillus timonensis* strain LK-DZ15.

Purification step	Total activity (units)[a,b,c] × 10^3	Total protein (mg)[b,d]	Specific activity (U/mg of protein)[b]	Activity recovery rate (%)	Purification factor (-fold)
Crude extract	5750 ± 90	2875 ± 33	2000 ± 25	100	1
$(NH_4)_2SO_4$ fractionation (40–70%)-Dialysis	5060 ± 81	1126 ± 18	4494 ± 77	88	2.2
FPLC (UNO Q-12)	4312 ± 65	389 ± 10	11,085 ± 105	75	5.5
HPLC (ZORBAX PSM 300 HPSEC)	3162 ± 40	24 ± 1	131,750 ± 710	55	66

[a] From 500 mL of a 44 h culture of *Paenibacillus timonensis* strain LK-DZ15.
[b] The experiments were conducted 3 times and ± standard deviations are reported.
[c] One unit (U) of chitinase activity was defined as the amount of enzyme releasing 1 μmol of Glc-NAc per min at 80 °C with colloidal chitin as substrate.
[d] Amounts of protein were estimated by the Bradford method [78].

Table 2
Comparison between the properties of chitinase ChiA-Pt70 and those of other common chitinases and GH-18 chitinases family from literature.

Chitinase	Microorganism	pH opt.	Optima temp. (°C)	Half-life time (min)[a]	MW (kDa)[b]	Reference
ChiA-Pt70	*Paenibacillus timonensis* strain LK-DZ15	4.5	80	120 (90 °C)	70	This study
PbChi70	*Paenibacillus barengoltzii* strain CAU904	5.5	55	29 (65 °C)	70	[26]
PeChiA68	*Paenibacillus elgii* strain HOA73	7	50	900 (50 °C)	68	[79]
Chi 56	*Paenibacillus* sp. strain D1	5	50	23 (80 °C)	56	[22]
ChiPp	*Paenibacillus pasadenensis* strain NCIM 5434	10	37	NR	35	[24]
ChiPi	*Paenibacillus illinoisensis* strain KJA-424	5	60	NR	54	[23]
ChiPt	*Paenibacillus thermoaerophilus* strain TC22-2b	4	60	1.5 (50 °C)	48	[64]
ChiA	*Serratia marcescens* strain QM B1466	6.4	50	NR	NR	[12]
ChiA	*Serratia marcescens* strain 2170	6	60	NR	60	[40]
Chit62	*Serratia marcescens* strain B4A	6	55	NR	62	[51]
ChiA	*Serratia marcescens* strain BJL200	5–6	50–60	400 (37 °C)	65	[76]
Chi 52	*Serratia marcescens* strain KCTC2172	5.5	45	1.5 (50 °C)	52	[80]
ChiA-Mt45	*Melghiribacillus thermohalophilus* strain Nari2A^T	3.5	90	480 (90 °C)	45	[27]
ChiA-Hh59	*Hydrogenophilus hirschii* strain KB-DZ44	5	85	240 (90 °C)	59	[28]
ChiA-65	*Bacullus licheniformis* strain LHH100	4	75	240 (90 °C)	65	[29]
Chi	*Bacullus licheniformis* strain MB-2	6	70	80 (60 °C)	67	[14]
Chi 72	*Bacullus licheniformis* strain SK1	6–8	55	90 (60 °C)	72	[81]
Chi	*Bacillus* sp. strain NTCU2	7	60	30 (60 °C)	36.5	[82]
Chi-L	*Bacillus* strain MH-1	6.5	75	10 (80 °C)	71	[83]
Chi-M	*Bacillus* strain MH-1	5.5	65	10 (70 °C)	62	[83]
Chi-S	*Bacillus* strain MH-1	5.5	75	10 (80 °C)	53	[83]
Chi	*Streptomyces lunalinharesii* strain RCQ1071^T	8	40	60 (60 °C)	70	[84]
Chi 30	*Streptomyces thermoviolaceus* strain OPC-520	4	60	30 (60 °C)	30	[85]
Chi-F1	*Pseudomonas aeruginosa* strain K-187	8	50	50 (10 °C)	30	[86]
Chi-F2	*Pseudomonas aeruginosa* strain K-187	7	40	50 (10 °C)	30	[86]
SsChi50	*Scorpaena scrofa*	4	75	480 (90 °C)	50	[38]

[a] NR, Not reported.
[b] MW, Molecular weight.

Table 3
Alignment of the NH_2-terminal amino acid sequence of the purified chitinase ChiA-Pt70 from *Paenibacillus timonensis* strain LK-DZ15 with the sequences of other *Paenibacillus* GH-18 chitinases family.

Enzyme	Origin	NH_2-terminal amino acid[a]	Identity (%)[b]
ChiA-Pt70 (This work)	*Paenibacillus timonensis* strain LK-DZ15	AAAWAPNTSYKWYDLVSYGGSEYQCLQ	–
PtChiA71 (WP_088,831,691)	*Paenibacillus elgii* strain NBRC 100,335	AAAWAPNTSY**KNGDIVTYGGSDYQCLQ**	81
PeChiA68 (ARU77129)	*Paenibacillus elgii* strain HOA73	AAAWAPNTSY**KAGDIVSYGGSDYQCLQ**	79
PeChi71 (WP_025,849,605)	*Paenibacillus ehimensis* strain NBRC 15,659	AAAWAPNTSY**KVGDIVTYGGSDYQCLQ**	75
PcChi71 (WP_042,225,994)	*Paenibacillus chitinolyticus* strain NBRC 15,660	AA**Q**WAPNT**AYKAK**DLVSY**NGVTYECLQ**	70
PaChi64 (WP_021,252,861)	*Paenibacillus alvei* strain DSM 29	AA**PWSPNTAYKTG**DLVSY**SGKTYACIQ**	59
PdChiD (EHQ62912)	*Paenibacillus dendritiformis* strain C454	AAAWAP**GVSYAADD**LVSY**QGKIYRCRQ**	57

[a] Amino acid sequences for comparison were obtained using the program BLASTP (NCBI, NIH, USA) database.
[b] Residues not identical with ChiA-Pt70 are indicated in black box. The GenBank accession number is in parentheses.

Paenibacillus barengoltzii strain SAFN-125^T (GenBank accession n°.: DQ124699). Those sequences were imported into MEGA software package version 4.1 and aligned. Phylogenetic trees were then constructed (Fig. 1B) and the findings further confirmed that the LK-DZ15 strain (GenBank accession n°.: **MK734103**) was closely related to those of the *Bacillus* strains. All the results obtained strongly suggested that this isolate should be assigned as *Paenibacillus timonensis* strain LK-DZ15.

3.3. 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 sulphate precipitation (40–70%) followed by sequential column chromatographies on FPLC and HPLC. Fractions

Table 4
Effects of various reagents on purified chitinase ChiA-Pt70 from *Paenibacillus timonensis* strain LK-DZ15 with colloidal chitin as substrate.

Chemical reagent/metallic ions[a]	Amino acid involved/material	Concentration (mM)	Relative activity (%)[b]
None	–	–	100 ± 2.5
p-CMB	Cysteine	2	0 ± 0.0
DTNB	Cysteine	2	0 ± 0.0
NEM	Cysteine	2	0 ± 0.0
IAM	Cysteine	1	11 ± 0.5
IAA	Cysteine	1	13 ± 0.6
LD-DTT	Cysteine	1	15 ± 0.6
β-ME	Cysteine	5	10 ± 0.5
TNBS	Lysine	2	99 ± 2.5
PMSF	Serine	5	101 ± 2.5
EDC	Carboxylic amino acids	2	115 ± 2.7
DEP	Histidine	1	98 ± 2.5
NBS	Typtophane	2	101 ± 2.5
NAI	Tyrosine	5	102 ± 2.5
Ca^{2+}	$CaCl_2$	5	235 ± 5.0
Mn^{2+}	$MnCl_2$	5	175 ± 3.6
Mg^{2+}	$MgCl_2$	5	140 ± 2.8
Co^{2+}	$CoCl_2$	5	111 ± 2.6
Cu^{2+}	$CuCl_2$	5	103 ± 2.5
Zn^{2+}	$ZnCl_2$	5	122 ± 3.0
Ba^{2+}	$BaCl_2$	5	85 ± 1.8
Fe^{2+}	$FeCl_2$	5	90 ± 2.0
Ag^{2+}	$AgNO_3$	5	16 ± 0.6
Al^{2+}	$AlCl_2$	5	77 ± 1.5
Cd^{2+}	$CdCl_2$	5	0 ± 0.0
Hg^{2+}	$HgCl_2$	5	0 ± 0.0
Ni^{2+}	$NiCl_2$	5	0 ± 0.0

[a] Incubation with 50 µg of purified enzyme at 80 °C at pH 4.5 for 30 min.
[b] Values represent means of 3 replicates, and ± standard deviations are reported.

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 (Fig. 2A). Purification to homogeneity was achieved using a ZORBAX PSM 300 HPSEC using HPLC system. The fraction containing the chitinase activity was eluted at Rt of 8.979 min (Fig. 2B).

The results of the purification procedure are summarized in Table 1. Enzyme purity was estimated to be 66-fold greater than that of the crude extract. The purified enzyme had a specific activity of 131,750 U/mg, with a yield of about 55%. In fact, the specific activity displayed by ChiA-Pt70 was significantly high compared to those previously reported by the authors for other chitinases. For example, the chitinase ChiA-Mt45 from *Melghiribacillus thermohalophilus* strain Nari2A^T showed a specific activity of 70,500 U/mg [27], the chitinase ChiA-Hh59 produced by *Hydrogenophilus hirschii* strain KB-DZ44 showed a specific activity of 21,600 U/mg [28] and the chitinase ChiA-65 produced by *Bacillus licheniformis* strain LHH100 showed a specific activity of 7869.5 U/mg [29]. This high level of its specific activity confirmed the potential prospects of ChiA-Pt70 in various biotechnological and industrial bioprocesses.

3.4. 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. 2C) and clear chitinase activity (Fig. 2D). MALDI–TOF/MS analysis confirmed that the purified ChiA-Pt70 had an exact molecular mass of 70,166.11 kDa (Fig. 2E). These results suggested that ChiA-Pt70 was a monomeric protein comparable to those previously reported for other bacterial chitinases [28,29,44–50]. Considerable variation in the molecular weight of chitinase had been reported earlier *viz.* 65 kDa for ChiA-65 from *Bacillus licheniformis* strain LHH100 [29], 62 kDa for Chit62 from *Serratia marcescens* strain B4A [51], 60 kDa for *Moritella marina* strain ATCC 15381^T [52], 59 kDa for ChiA-Hh59 produced by *Hydrogenophilus hirschii* strain KB-DZ44 [28], 55 kDa for ChiA from *Sanguibacter antarcticus* strain KOPRI 21,702 [53], 51.66 kDa for ChiA from *Pseudomonas* sp. strain TXG6-1 [54], 45 kDa for ChiA-Mt45 from *Melghiribacillus thermohalophilus* strain Nari2A^T [27], and 35 kDa for ChiA from *Paenibacillus pasadenensis* strain NCIM 5434 [24]. Generally, the molecular weight of chitinase was ranged from 30 to 72 kDa (Table 2).

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

The twenty seven NH_2-terminal amino acid residues were determined to be AAAWAPNTSYKWYDLVSYGGSEYQCLQ (Table 3). 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 100,335 and the chitinase PeChiA68 from *Paenibacillus elgii* strain HOA73 (Table 2). 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 15,659, PcChi71 from *Paenibacillus chitinolyticus* strain NBRC 15,660, 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.

3.6. Biochemical characterization of the purified chitinase

3.6.1. 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 β-ME (Table 4). This indicates the presence of sulfhydryl groups on active site of the enzyme, as confirmed by total inhibition observed in the presence of mercuric ion. It is well-known that the formation of disulfide bonds is critical for stabilizing protein structures and maintaining protein functions [55]. NEM is a widely used alkylation reagent to quench free thiols in disulfide bond analysis due to its thiol specificity and stability below pH 7 [56,57]. It was also found to be more efficient than IAM or IAA in terms of reaction time and reagent amount needed [57,58]. However, the 3D structure of proteins may hinder the access of NEM and decrease the blocking efficiency [59]. The chitinases from *Melghiribacillus thermohalophilus* strain Nari2A^T [27], *Bacillus licheniformis* strain LHH100 [29], *Hydrogenophilus hirschii* strain KB-DZ44 [28], and *Streptomyces* sp. strain M − 20 [60] 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 [27], ChiA-65 from *Bacillus licheniformis* strain LHH100 [29], and ChiA-Hh59 from *Hydrogenophilus hirschii* strain KB-DZ44 [28].

The effects of various metallic ions at 2 and 10 mM on the chitinase activity are shown in Table 3. The obtained results showed that the test with 2 mM was retained since there was no difference with that of

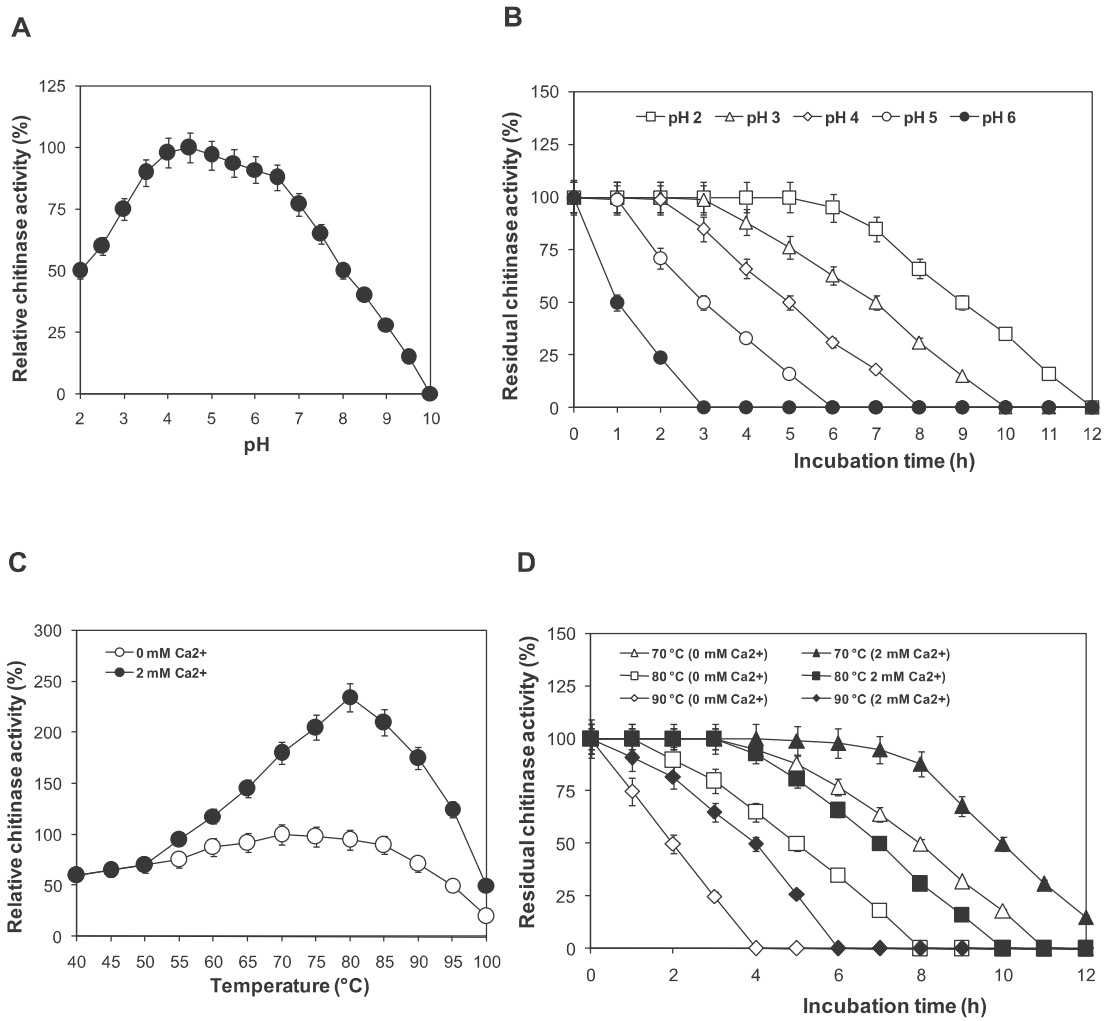

Fig. 3. Effects of pH on the activity **(A)** and stability **(B)** of the purified chitinase ChiA-Pt70 from *Paenibacillus timonensis* strain LK-DZ15. The activity of the enzyme at pH 4.5 was taken as 100%. Buffer solutions used for pH activity and stability are presented in Section 2. Effects of the thermoactivity **(C)** and the thermostability **(D)** 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.

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 (Table 4). 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 [61]. It strongly inhibits chitinases from different genera [46]. Report on the effect of metallic ions on chitinases is quite divers [29,44,62].

3.6.2. *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. 3A). 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 [63] and *Paenibacillus thermoaerophilus* strain TC22-2b [64] 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 [29]. However, the chitinases of *Paenibacillus* sp. strain D1 [22], *Paenibacillus illinoisensis* strain KJA-424 [23], *Streptomyces violaceusniger* strain MTCC 3959 [65], and *Hydrogenophilus hirschii* strain KB-DZ44 [28] show the optimum pH at 5. Otherwise, the chitinases of *Paenibacillus pasadenensis* strain NCIM 5434 [24] and *Sanguibacter antarcticus* strain KOPRI 21,702 [53] 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 [40,66].

The pH stability profile of purified ChiA-Pt70 indicated that this enzyme was highly stable in the pH range of 2–6 (Fig. 3B). 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.

3.6.3. *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. 3C). The optimal temperature of ChiA-Pt70 was found to be much

Table 5
Substrate specificities of the purified chitinase ChiA-Pt70 from *Paenibacillus timonensis* strain LK-DZ15 for various substrates.

Substrate	Concentration (mg/mL)	Monitoring wavelength (nm)	Specific activity (U/mg of protein)[a]	Relative chitinase activity (%)[b]
Colloidal chitin	50	550	131,750 ± 710	100 ± 2.5
Chitin azure	50	550	129,115 ± 705	98 ± 2.5
Glycol chitin	50	550	125,162 ± 690	95 ± 2.4
Chitin	50	550	39,525 ± 265	30 ± 0.9
Glycol chitosan	50	550	119,892 ± 684	91 ± 2.0
Chitibiose	50	550	0 ± 0.0	0 ± 0.0
Chitotriose	50	550	115,940 ± 665	88 ± 1.8
Chito-oligosaccharide[c]	50	550	104,082 ± 657	79 ± 1.4
Xylan	50	550	65,875 ± 493	50 ± 1.1
Amylose	40	550	0 ± 0.0	0 ± 0.0
Carboxymethyl cellulose	40	550	ND	ND
Cellobiose	40	550	ND	ND
Avicel	40	550	0 ± 0.0	0 ± 0.0
Laminarin	40	550	0 ± 0.0	0 ± 0.0
p-NP-(GlcNAc) (G-P)	10	420	ND	ND
p-NP-$(GlcNAc)_2$ (G-G-P)	10	420	ND	ND
p-NP-$(GlcNAc)_3$ (G-G-G-P)	10	420	185,767 ± 790	141 ± 2.8
p-NP-$(GlcNAc)_4$ (G-G-G-G-P)	10	420	408,425 ± 999	310 ± 5.5
p-NP-$(GlcNAc)_5$ (G-G-G-G-G-P)	10	420	856,375 ± 3250	650 ± 7.7

G, GlcNAc. P, *p*-NP. ND, the enzyme activity was not detected. Zero (0), indicates that no product was generated in reaction mixture with the substrate.
[a] All measurements were carried out in buffer A (pH 4.5) at 80 °C. Values represent maximum activity obtained after 12 h of incubation for the natural substrates and 6 h for the synthetic substrates, and the optimum substrate concentration.
[b] Activity is expressed as a percentage of enzyme activity under standard conditions using colloidal chitin or *p*-NP-$(Glc\text{-}NAc)_2$. Values represent means of 3 replicates, and ± standard deviations are reported.
[c] Chito-oligosaccharides are a mixture of chitotetraose, chitopentaose, and chitohexaose.

higher than those of most other reported bacterial chitinases with optimal temperatures in the range of 28–60 °C [24,52,62] and 75–80 °C [29]. While the chitinase from *Bacillus thuringiensis* subsp. *kurstaki* strain HBK-51 showed maximum activity at 110 °C [67].

The thermostability profile of ChiA-Pt70 is presented in Fig. 3D. 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 [29,68]. The thermostability of ChiA-Pt70 was higher than several other previously reported chitinases [29,44,51,62].

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.

3.7. Kinetic measurements of the purified chitinase

3.7.1. Substrate specificity of ChiA-Pt70

The specific activities of ChiA-Pt70 towards different substrates are presented in Table 5. Among the polysaccharides, ChiA-Pt70 showed highest specific activity towards colloidal chitin (131,750 U/mg) followed by chitin azure (129,115 U/mg), but displayed less activity against xylan (65,875 U/mg) and chitin (39,525 U/mg). No chitinolytic activities were measured towards the other tested substrates including chitibiose, amylose, avicel, and laminarin. This suggests that the chitin binding domain of ChiA-Pt70 processes a strong capacity for particulate chitin, which is similar to other bacterial chitinases [29,38,69–71]. Moreover, the enzyme was highly active towards *p*-NP-$(GlcNAc)_5$ (G-G-G-G-G-P) (856,375 U/mg) followed by *p*-NP-$(GlcNAc)_4$ (G-G-G-G-P) (408,425 U/mg), and *p*-NP-$(GlcNAc)_3$ (G-G-G-P) (185,767 U/mg), but displayed no activity towards *p*-NP-$(GlcNAc)_2$ (G-G-P) and *p*-NP-(GlcNAc) (G-P) (Table 5). This behavior was already described for other chitinases [29,38,69–71].

3.7.2. Determination of the kinetic parameters

The ChiA-Pt70, ChiA-Mt45, ChiA-Hh59, ChiA-65, Chitodextrinase®, and *N*-acetyl-β-glucosaminidase® enzymes exhibited the classical kinetics of Michaelis-Menten for the two substrates used (Fig. 4). The K_m and k_{cat} values of ChiA-Pt70 were 0.611 mg colloidal chitin/mL and 87,800 s^{-1}, respectively. The order of the catalytic efficiency (k_{cat}/K_m) values of each enzyme was almost the same, i.e., colloidal chitin < *p*-NP-$(Glc\text{-}NAc)_3$ (G-G-G-P) (Table 6). When *p*-NP-$(Glc\text{-}NAc)_4$ (G-G-G-G-P) was used as a synthetic substrate, ChiA-Pt70 was noted to exhibit k_{cat}/K_m values that were 59.3, 41.3, 34.2, 13.8, and 3.8 times higher than that of ChiA-65, *N*-acetyl-β-glucosaminidase®, Chitodextrinase®, ChiA-Hh59, and ChiA-Mt45, respectively (Table 6).

3.8. Enzymatic performance of the purified chitinase

3.8.1. Effect of organic solvents on the stability of the purified ChiA-Pt70

In this study, various organic solvents (with different log *P* values) were examined for their effects on the stability of the purified ChiA-Pt70, ChiA-Mt45, ChiA-Hh59, ChiA-65, Chitodextrinase®, and *N*-acetyl-β-glucosaminidase® chitinases (Fig. 5A). The stability shown by ChiA-Pt70 in the presence of DMF (195%), chloroform (177%), DMSO (162%), *n*-hexane (155%), cyclohexane (131%), and *iso*-propanol (120%), were higher than those of the other used comparative enzymes. When compared to the control, ChiA-Mt45, ChiA-65, ChiA-Hh59, Chitodextrinase®, and *N*-acetyl-β-glucosaminidase® were noted to retain at least 150, 104, 94, and 8% of their activities after 24 h of incubation in the presence of cyclohexane as a hydrophobic solvent and 130, 109, 120, and 116% of their activities in the presence of ethanol as a hydrophilic solvent, respectively. By contrast, the tested chitinases were noted to be completely deactivated by acetonitrile and ethyl acetate, which have previously been reported to be quite harmful polar aprotic solvents to other solvent-stable enzymes. The solvent stability of ChiA-Pt70 chitinase was much higher than that reported for several other chitinases, such as: chitinases from *Melghiribacillus thermohalophilus* strain Nari2A^T [27], *Hydrogenophilus hirschii* strain KB-DZ44 [28], *Lecanicillium lecanii* strain 43H [45], and *Aeromonas hydrophila* strain SBK1 [72].

The organic solvent stability of the purified ChiA-Pt70 chitinase clearly indicated that it could be very useful for both types of reactions,

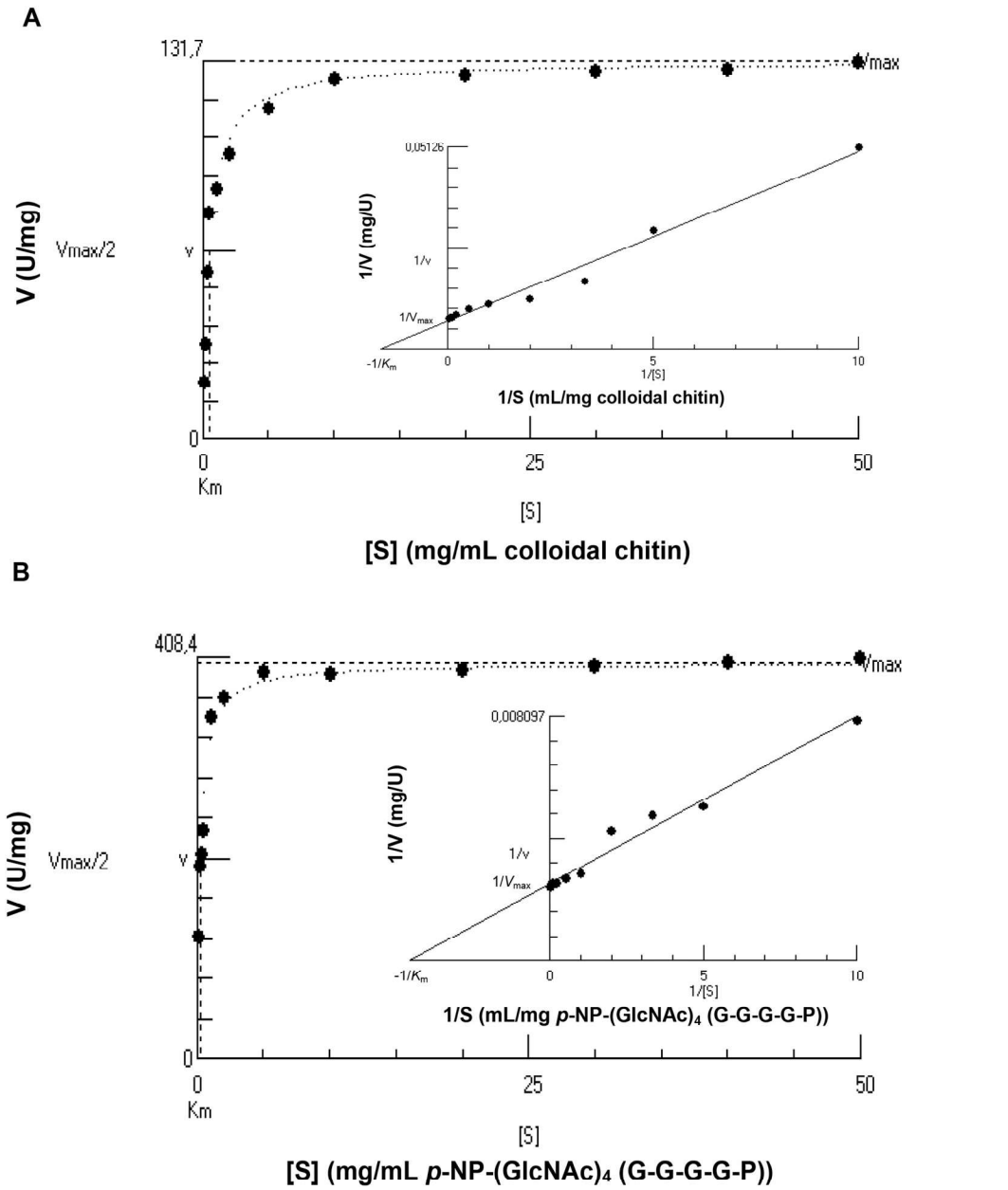

Fig. 4. Determination of K_m and V_{max} of the purified ChiA-Pt70 enzyme from *Paenibacillus timonensis* strain LK-DZ15 using colloidal chitin **(A)** and *p*-NP-$(GlcNAc)_4$ (G-G-G-G-P) **(B)** as substrates. Chitinase activities were carried out in triplicate and were measured at 70 °C for 5 min in assay buffer A supplemented with 5% (v/v) DMSO and 1% (v/v) Triton X-100 at pH 4. Inset: the corresponding double reciprocal plots of the purified chitinase ChiA-Pt70. The linear regression gives an equation with $r^2 = 0.960$.

Table 6
Kinetic parameters of the purified chitinase ChiA-Pt70 from *Paenibacillus timonensis* strain LK-DZ15 toward natural and synthetic substrates.

Substrate	Enzyme	K_m (mg/mL)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m (mL mg s^{-1})
Colloidal chitin	ChiA-Pt70	0.611 ± 0.07	131,700 ± 710	87,800 ± 610	143,700 ± 765
	ChiA-Mt45	0.253 ± 0.01	70,500 ± 510	47,000 ± 302	185,770 ± 790
	ChiA-Hh59	0.298 ± 0.01	21,600 ± 190	14,400 ± 131	48,322 ± 311
	Chitodextrinase*	0.457 ± 0.05	12,056 ± 125	8037 ± 120	17,586 ± 275
	N-acetyl-β-glucosaminidase*	0.491 ± 0.09	11,244 ± 108	7496 ± 103	15,267 ± 136
	ChiA-65	0.385 ± 0.03	7500 ± 104	5000 ± 74	12,987 ± 110
p-NP-$(GlcNAc)_4$ (G-G-G-G-P)	ChiA-Pt70	0.306 ± 0.01	408,400 ± 999	272,270 ± 825	889,770 ± 3500
	ChiA-Mt45	0.404 ± 0.03	139,590 ± 751	93,060 ± 655	230,346 ± 801
	ChiA-Hh59	0.413 ± 0.04	54,000 ± 380	36,000 ± 244	64,503 ± 477
	Chitodextrinase*	0.710 ± 0.08	38,855 ± 253	25,904 ± 201	26,000 ± 213
	N-acetyl-β-glucosaminidase*	0.749 ± 0.09	31,295 ± 240	20,864 ± 177	21,509 ± 182
	ChiA-65	0.601 ± 0.07	13,515 ± 250	9010 ± 130	14,992 ± 135

Values represent mean of 3 replicates, and ± standard deviations are reported.

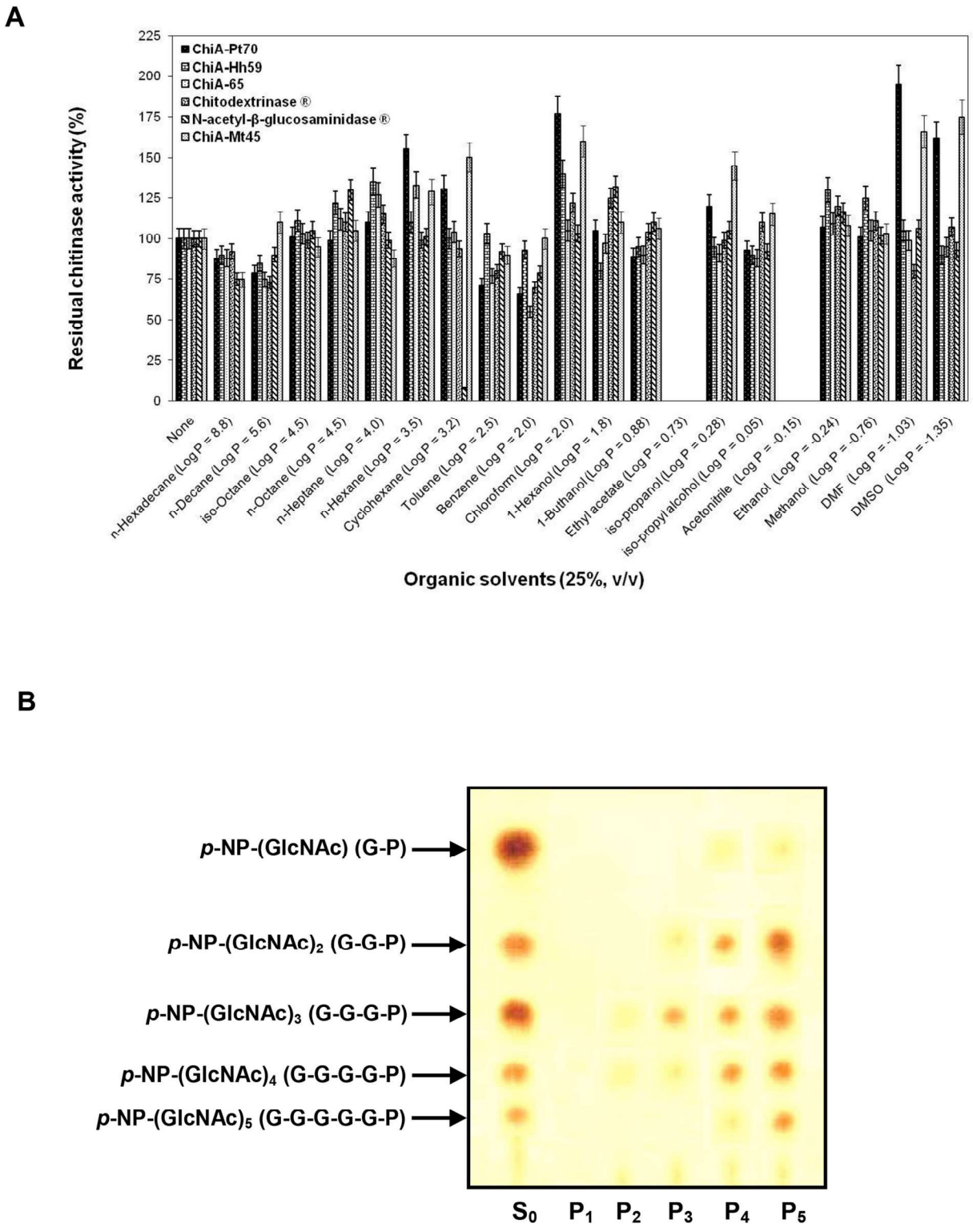

Fig. 5. (A) Effect of organic solvents on the stability of the purified ChiA-Pt70, ChiA-Mt45, ChiA-Hh59, ChiA-65, Chitodextrinase®, and *N*-acetyl-β-glucosaminidase® chitinases. Various organic solvents, with different Log *P* values (25%, v/v), were tested at 50 °C and with shaking at 160 strokes per min for 24 h to evaluate their effects on chitinase stability. The residual chitinolytic activities were assayed under the same conditions of each enzyme. The activity of the enzyme without any organic solvent was taken as 100%. The activity is expressed as a percentage of activity level in the absence of organic solvents. Each point represents the mean of three independent experiments. **(B)** Thin-layer chromatogram analysis showing hydrolysis product of colloidal chitin by the purified chitinase ChiA-Pt70 from *Paenibacillus timonensis* strain LK-DZ15 for various times. Lane S_0 indicates standard *N*-acetyl-chito-oligosaccharides ranging from monomer (G_1) to pentamer (G_5). Lanes P_1 indicates unhydrolyzed substrate. Lanes P_2 through P_5 indicates hydrolysates obtained after 1, 6, 12, and 24 h of the reaction, respectively.

i.e., a reaction with only aqueous solution and a reaction with aqueous solution and added solvents (co-solvents). The stability of the chitinase ChiA-Pt70 at varied organic solvents assures its employment in industrial sectors.

3.8.2. Hydrolytic products from colloidal chitin

The products of colloidal chitin hydrolysed by the purified chitinase ChiA-Pt70 from *Paenibacillus timonensis* strain LK-DZ15 were determined by TLC. As shown in Fig. 5B the purified chitinase ChiA-Pt70 completely cleaved colloidal chitin into a mixture of dimer *p*-NP-$(GlcNAc)_2$ (G-G-P), trimer *p*-NP-$(GlcNAc)_3$ (G-G-G-P), tetramer *p*-NP-$(GlcNAc)_4$ (G-G-G-G-P), and pentamer *p*-NP-$(GlcNAc)_5$ (G-G-G-G-G-P). A trace amount of monomer (GlcNAc) was observed at the end of reaction. The results mentioned above suggesting that the chitinase ChiA-Pt70 acted as an *endo*-splitting enzyme as demonstrated for other bacterial chitinases [29,73,74]. Recent studies suggest that GH degree of *endo*-activity is best assessed as a probability of *endo*-mode initiation where concentrations of both insoluble reducing groups (*endo*-initiation) and reducing end groups (*exo*-initiation) upon substrate degradation is determined [75]. Still, the observation of chito-oligosaccharides with degree of polymerization higher than 2 is indicative of ChiA-Pt70 having *endo*-activity as ChiA and ChiC from *Serratia marcescens* [75–77].

4. Conclusions

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. Compared to ChiA-Mt45, ChiA-Hh59, ChiA-65, Chitodextrinase®, and *N*-acetyl-β-glucosaminidase® chitinases, ChiA-Pt70 showed high catalytic efficiency. These features collectively suggest its potential relevance for commercial exploitation in the future. Considering the attractive properties and attributes of the ChiA-Pt70 enzyme, further studies are needed to explore the molecular structure of its encoding gene and regulation region and investigate its structure-functions relationship using site-directed mutagenesis and 3D structure modeling.

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