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Identification and characterization of a highly chitinase-producing *Paenibacillus timonensis* strain LK-DZ15 isolated from Djurdjura Mountains in Kabylia, Algeria

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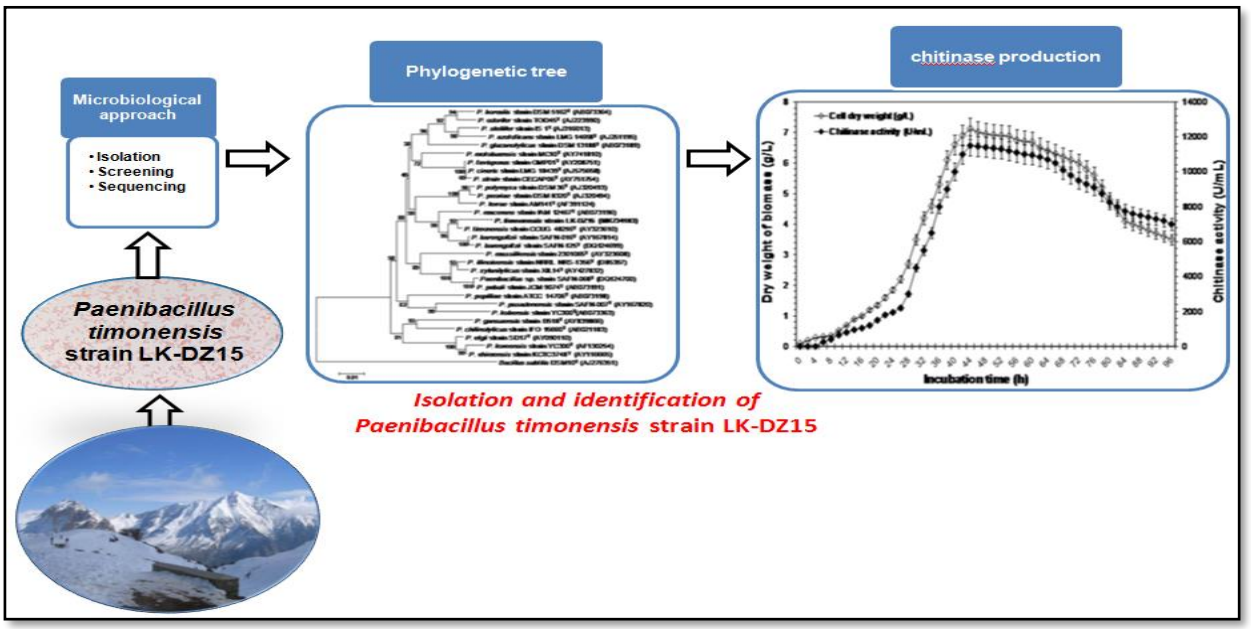
Graphical Abstract



Abstract

A novel bacterial strain was isolated from the highest summit of the Djurdjura Mountains in Kabylia (Algeria) at altitudes of about 23 km. For a long time, scientists have investigated in familiar world to identify novel microbial biocatalysts. However, the mountain soil has been shown as an almost entire reserve of novel enzymes with interesting properties for industrial and environmental applications. *Paenibacillus* sp. as a genus of facultative anaerobic bacteria is widespread in nature. Many species of this genus produce different enzymes used in biodegradation, textiles, bakery, food industry, stationery, biopharmaceutical industries and in many other domains. Thus, the strain LKDZ15 was isolated from Tikjda, in the Djurdjura Mountains, Algeria. The identification of this newly isolated bacterium was carried out using morphological, physiological, and biochemical characteristics. In addition, the *16S* rDNA gene was also amplified and sequenced. All the data obtained with regards to the physiological and biochemical properties of the isolate, confirmed that the LKDZ15 strain belonged to the *Paenibacillus* genus. Moreover, the nucleotide sequence and blast analyses confirmed that the LKDZ15 strain (GenBank accession no.: MK734103) was closely related to those of the *Paenibacillus* strains. All the results obtained strongly suggested that this new isolate should be assigned as *Paenibacillus timonensis* strain LKDZ15.

1. Introduction

Various chitinases have been isolated from some bacterial strains such as *Serratia marcescens* [1], *Bacillus cereus* [2], *Bacillus licheniformis* [3], and *Stenotrophomonas maltophilia* [4]. The GHs are categorized into families (18 and 19) according to their sequences in the CAZy database (www.cazy.org) [5]. Those belonging to family 18 are distributed among bacteria, plants, animals, and other organisms [6]. On the other hand, family 19, are present mainly in higher-order plants and are reported to have strong antibacterial properties [7]. Chitinases are classifed 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 (GlcNAc2). *Endo*-chitinases randomly cleave β-1,4- glycosidic bonds of chitin, whereas *N*-acetylglucosaminases hydrolyse GlcNAc2 into GlcNAc or produce GlcNAc from the non-reducing end of *N*-acetyl-chito-oligosaccharides [8]. 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 [9]. Several species from this genus have been found to produce chitinases such as *Paenibacillus* sp. [10–12], *Paenibacillus illinoisensis* strain KJA-424 [13], *Paenibacillus pasadenensis* strain NCIM 5434 [14], and *Paenibacillus barengoltzii* strain CAU904 [15, 16]. However, to the best of the authors' knowledge, no report is available regarding production, purifcation, and characterization of chitinase from *Paenibacillus timonensis*.

2. Materials and Methods

2.1. Preparation of colloidal chitin

Colloidal chitin was prepared according to the method of Roberts and Selitrennikoff [17] 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.

2.2. 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 (2,308 meters), 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 (2,500 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.

2.3. 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* [18]. The 16S rRNA gene was amplified by PCR

using forward 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse 1525R (5' AAGGAGGTGATCCAAGCC-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^- *supE44 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 Lysogeny-Broth (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. [19].

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.

2.4. Nucleotide sequence accession number

The nucleotide sequence data of 16S rRNA gene 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.

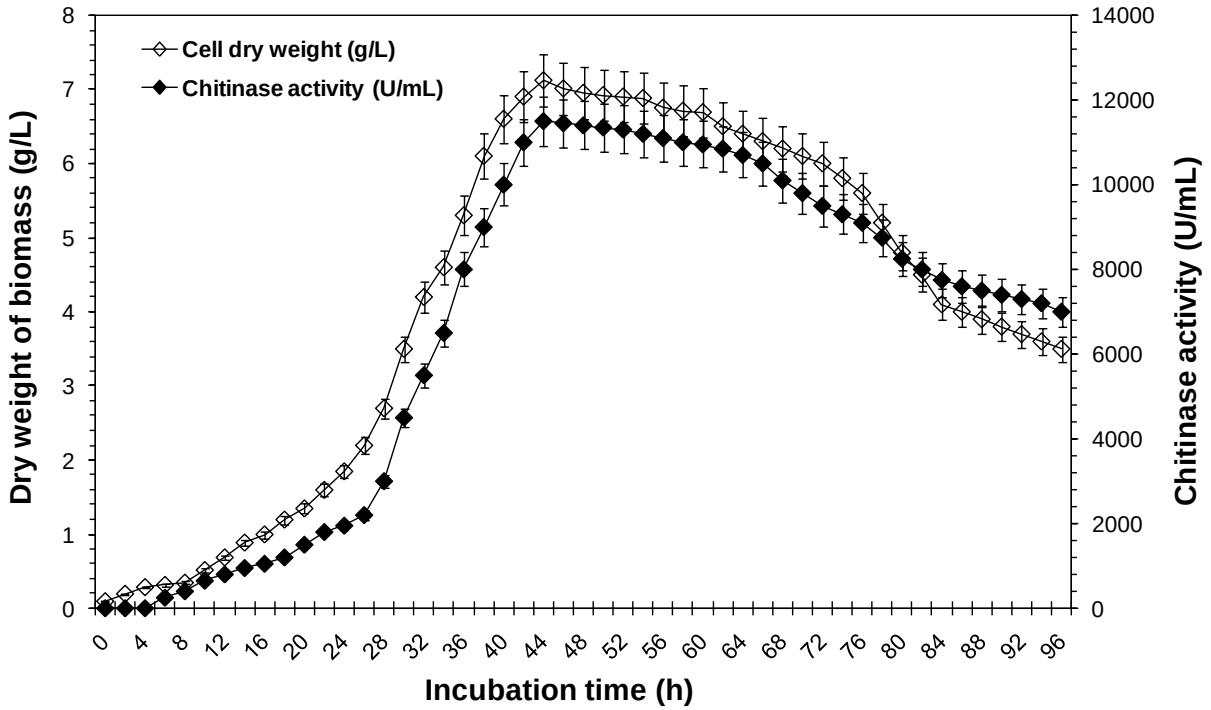


Fig. 1. Time course of *Paenibacillus timonensis* strain LK-DZ15 cell growth (Δ) and chitinase production (▲). Cell growth was monitored by measuring the absorbance at 600 nm and was converted to cell dry weight (g/L). Each point represents the mean of three independent experiments.

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 [20,23].

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 *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[21-22].

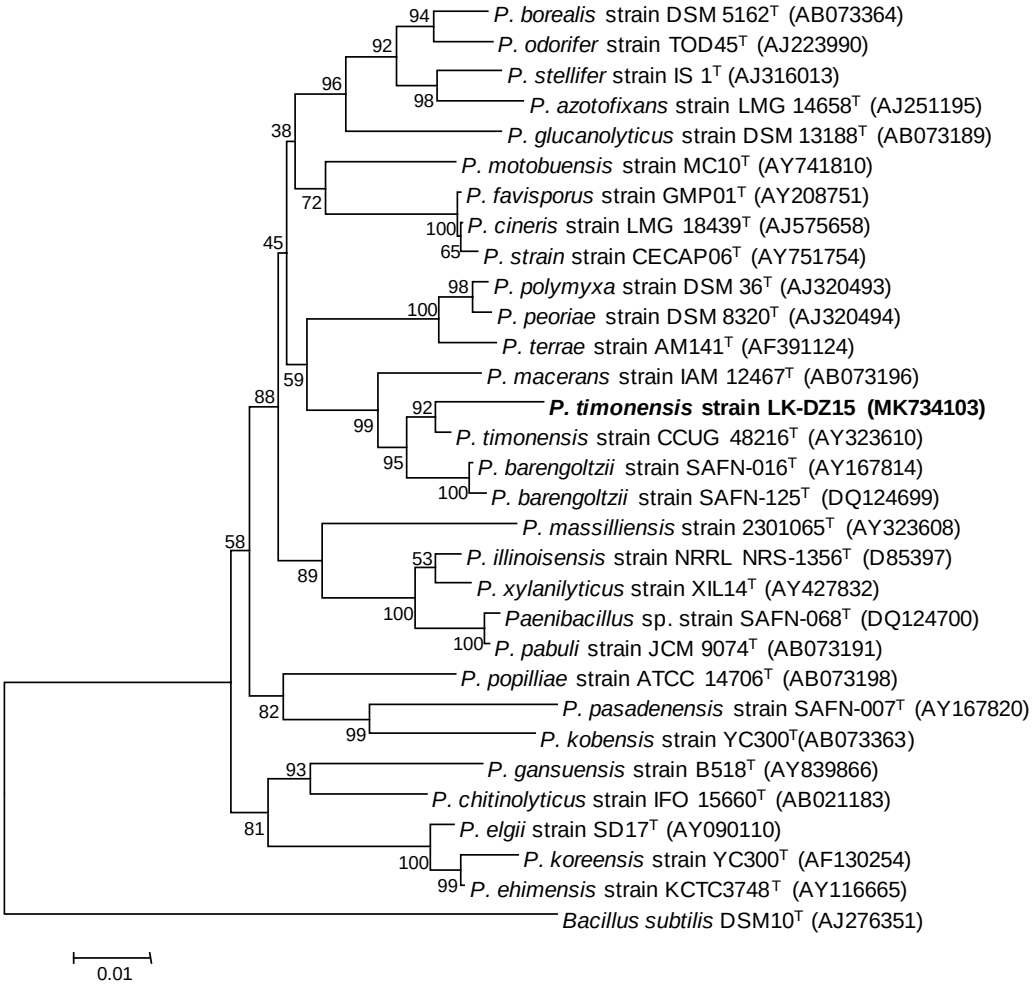


Fig.2. Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain LHH100 within the radiation of the genus *Paenibacillus*. The sequence of *Bacillus subtilis* strain DSM 10^T (GenBank accession n° AJ276351) was chosen as an outgroup. Bar, 0.01 nt substitutions per base. Numbers 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.

4. Conclusion

All the data obtained with regards to the physiological and biochemical properties of the isolate, confirmed that the LKDZ15 strain belonged to the *Paenibacillus* genus. Moreover, the nucleotide sequence and blast analyses confirmed that the LKDZ15 strain (GenBank accession no.: **MK734103**) was closely related to those of the *Paenibacillus* strains. All the results obtained strongly suggested that this new isolate should be assigned as *Paenibacillus timonensis* strain LKDZ15. This new isolated strain LK-DZ15 produced significantly high amounts of extracellular chitinase.

5. References

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