Polish Journal of Microbiology
2021, Vol. 70, No 3, 297–304
https://doi.org/10.33073/pjm-2021-026

ORIGINAL PAPER

Hydrolytic Enzymes Producing Bacterial Endophytes of Some Poaceae Plants

GOKHAN DOGAN and BILGIN TASKIN*

Van Yuzuncu Yil University, Faculty of Agriculture, Department of Agricultural Biotechnology, Van, Turkey

Submitted 24 March 2021, revised 2 June 2021, accepted 7 June 2021

Abstract

Endophytic bacteria represent microorganisms that live during the whole life cycle within the tissues of healthy plants without causing any obvious signs of disease. In this study, the ability of 128 endophyte bacterial isolates from some cultivated and wild grain plants (Poaceae family) in Van, Turkey, were investigated in terms of producing several extracellular hydrolytic enzymes. It was demonstrated that lipases, proteases, amylases, cellulases, pectinases, and xylanases were produced by the bacteria with relative frequencies of 74.2%, 65.6%, 55.4%, 32%, 21.8%, and 7.8%, respectively. In addition, molecular identification of a certain number of isolates selected according to their enzyme-producing capabilities was performed by 16S rRNA gene sequencing using a next-generation sequencing platform. As a result of the analysis, the isolates yielded certain strains belonging to *Pseudomonas*, *Micrococcus*, *Paenibacillus*, *Streptococcus*, *Curtobacterium*, *Chryseobacterium*, and *Bacillus* genera. Also, the strain G117Y1T was evaluated as a member of potential novel species based on 16S rRNA sequencing results.

K e y w o r d s: endophytic bacteria, extracellular enzymes, 16S rRNA gene, Poaceae family, Illumina MiSeq

Introduction

Although endophytes have been widely defined as microorganisms that live in the tissues of healthy plants for all or part of their life cycle, recent studies have revised this definition to include all microorganisms, including pathogens that can colonize the internal tissues of plants (Hardoim et al. 2015; Compant et al. 2021). Endophytic bacteria (EBs) have been isolated and characterized from different plant parts, including roots, stems, leaves, seeds, fruits, tubers, ovules, and nodules of various plants such as agricultural crops, meadow plants, plants grown in extreme environments, wild, and perennial plants (Afzal et al. 2019). EBs can contribute to plant health and development like Plant Growth Promoting Rhizobacteria (PGPR). In general, PGPR and EBs directly or indirectly affect the growth and development of the plant. EBs stimulate plant growth through various mechanisms such as nitrogen fixation, phytohormone production, nutrient uptake, and providing the plant with tolerance to abiotic and biotic stresses (Kandel et al. 2017). These properties make these bacteria important for various biotechnological applications in agriculture. Also, they have the potential to produce a variety of secondary metabolites like alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols, and phenols with an application in agriculture, pharmaceutical and industrial biotechnology (Singh et al. 2017).

Microbial enzymes with high catalytic activities are used in many areas of the industry because they are more stable, cheaper, and can be obtained in large amounts by fermentation methods (Singh et al. 2016). Examples of industrial areas affected by discoveries of these enzymes include detergent agents, leather processing, degradation of xenobiotic compounds, food processing (bakery, meat, dairy, fruit, and vegetable products), pharmaceuticals (synthesis of pharmaceutical intermediates), biofuels (low-energy ethanol production process), and other enzyme related technologies (Singh et al. 2016). Although many bacterial isolates from various sources have been reported for the production of cellulase, protease, amylase, pectinase, lipase, asparaginase, etc., the studies involving the examination of endophytic bacteria in terms of biotechnological extracellular enzymes are relatively few (Carrim et al. 2006; Jalgaonwala and

* Corresponding author: B. Taskin, Van Yuzuncu Yil University, Faculty of Agriculture, Department of Agricultural Biotechnology, Van, Turkey; **e-mail: bilgintaskin@yyu.edu.tr**

Mahajan 2011; Khan et al. 2017). Therefore, endophytic bacteria can represent a new source of enzymes with different application potentials.

In addition to entry through openings and wounds, endophytic bacteria actively colonize plant tissues using hydrolytic enzymes, such as cellulase. It was proposed that cell wall-degrading enzymes such as cellulases, xylanases, and pectinases might be responsible for plant and microbe interactions and intercellular colonization of roots (Verma et al. 2001; Kandel et al. 2017). Therefore, more knowledge on their production is also needed to understand the relationship between endophytic bacteria and plants.

The aim of this study was to examine endophyte bacteria isolated from various cultivated and wild plants of Poaceae family in Van province, Turkey, in terms of their potential to produce industrially important proteases, amylases, lipases, cellulases, xylanases, and pectinases and to perform a phylogenetic affiliation of the strains possessing relatively high enzyme activity profiles by 16S rRNA gene sequence analysis.

Experimental

Materials and Methods

Bacterial isolates. In this study, endophyte bacteria isolated from some culture and wild grain plants (Poaceae family) stored at bacteriology laboratory, Department of Plant Protection, Faculty of Agriculture, Van Yuzuncu Yil University, were used. Endophytic bacteria had been isolated according to the method described by Ozaktan et al. (2015). The trituration technique with effective surface sterilization of the plant tissues was applied in this method. The plant species and tissues from which the bacteria were isolated were shown in Table I. All strains were grown either in Nutrient Broth (NB) broth (Difco, Detroit, MI, USA) or on Nutrient Broth agar plates at 25°C.

Determination of enzyme activities. The presence of the following enzymes were analyzed: amylases, lipases, cellulases, proteases, pectinases, and xylanases. The pure cultures of the isolates were inoculated onto solid diagnostic media by four isolated droplets. Enzyme Index (EI) is a practical tool that compares the enzymatic production of different isolates (Carrim et al. 2006; Jena and Chandi 2013). The EI for each enzyme was calculated at the end of a specific incubation time. EIs were calculated as a mean ratio of opaque zone diameter to colony diameter.

Amylase activity. The strains were inoculated onto nutrient agar supplemented with 1% (w/v) starch. After incubation for two days at 25°C, agar plate surfaces were treated with iodine solution, which allowed to observe unstained zone around active amylase colonies (Hankin and Anagnostakis 1975).

Lipase activity. Lipase activity was determined according to the method described by Hankin and

Table I
The plant species and the tissues from which the endophytic bacteria were isolated and enzymatic indexes (EIs) of hydrolytic enzymes of 16 strains selected for the 16S rRNA gene amplicon sequence analysis.

Isolate No	Host Plant	Plant Tissue	Protease	Lipase	Amylase	Cellulase	Pectinase	Xylanase
G90Y2	*Aegilops* sp.	Leaf	3.46 ± 0.15^{efg}	9.80 ± 0.20^{a}	2.14 ± 0.03^{de}	6.10 ± 0.16^{cd}	1.73 ± 0.03^{c}	–
G90S1	*Aegilops* sp.	Stem	2.94 ± 0.08^{ghi}	6.79 ± 2.01^{bc}	3.23 ± 0.09^{bc}	5.02 ± 0.27^{de}	–	–
G88K1	*Triticum aestivum* L.	Root	3.78 ± 0.06^{def}	1.90 ± 0.11^{e}	–	–	–	2.88 ± 0.38^{ns}
G83S3	*Triticum aestivum* L.	Stem	2.85 ± 0.05^{hi}	3.67 ± 0.15^{de}	3.91 ± 0.37^{ab}	4.40 ± 0.10^{e}	2.05 ± 0.05^{bc}	–
G105Y1	*Dactylis glomerata* L.	Leaf	7.29 ± 0.71^{a}	1.87 ± 0.34^{e}	3.03 ± 0.29^{bcd}	12.75 ± 1.38^{a}	3.81 ± 0.38^{a}	–
G105S1	*Dactylis* spp.	Stem	–	–	–	–	–	–
G100Y1	*Festuca* spp.	Leaf	3.40 ± 0.12^{fgh}	6.96 ± 0.54^{b}	2.18 ± 0.08^{de}	7.02 ± 0.46^{c}	–	–
G80K3	*Secale cereale* L.	Root	4.03 ± 0.17^{de}	–	3.05 ± 0.13^{bcd}	–	4.44 ± 0.90^{a}	–
G70K2	*Secale cereale* L.	Root	2.73 ± 0.34^{i}	7.24 ± 0.78^{b}	2.69 ± 0.04^{cd}	4.07 ± 0.13^{ef}	2.34 ± 0.18^{bc}	–
G42K2	Cultivated *Poaceae* spp.	Root	3.57 ± 0.20^{ef}	4.89 ± 0.22^{bcd}	2.68 ± 0.09^{cd}	2.66 ± 0.04^{f}	1.76 ± 0.14^{c}	–
G119Y1T	*Eremopoa* sp.	Leaf	–	4.37 ± 0.15^{cd}	1.29 ± 0.04^{e}	7.50 ± 0.00^{c}	–	–
G118S2T	*Eremopoa songarica* L.	Stem	4.22 ± 0.16^{cd}	4.46 ± 0.22^{cd}	1.69 ± 0.08^{de}	3.46 ± 0.19^{ef}	–	2.65 ± 0.41^{ns}
G117Y1T	*Eremopoa* sp.	Leaf	3.22 ± 0.13^{fghi}	6.32 ± 1.78^{bc}	2.81 ± 0.01^{cd}	2.46 ± 0.12^{f}	–	1.90 ± 0.27^{ns}
G116K1T	*Eremopoa songarica L.*	Root	4.68 ± 0.25^{bc}	1.91 ± 0.18^{e}	–	–	–	–
G113Y3	*Triticum aestivum* L.	Leaf	5.12 ± 0.07^{b}	3.15 ± 0.13^{de}	4.70 ± 0.17^{a}	9.77 ± 0.42^{b}	3.48 ± 0.29^{ab}	1.75 ± 0.25^{ns}
G107Y2	*Triticum aestivum* L.	Leaf	3.26 ± 0.09^{fghi}	7.33 ± 0.67^{b}	2.68 ± 0.27^{cd}	4.95 ± 0.30^{de}	–	–

Means of four replicates (Mean ± Std. Errors). Values within a column followed by different lowercase letters are significantly different ($p < 0.05$).
ns – not significant

Anagnostakis (1975) with minor modifications. The strains were inoculated onto the medium containing (g/l): Nutrient Broth 8 g, $CaCl_2$ H_2O 0.1 g, agar 15 g, pH 6.0, and 20 ml Tween 20. Tween 20 was separately added into the medium after sterilization. Cultures were incubated at 25°C for two or three days and the plates were kept at +4°C for 30 min. Variants showing opaque zone around colonies were evaluated as lipase positive.

Cellulase activity. Cellulase activity was determined by the method reported by Amore et al. (2015) with some modifications. The isolates were inoculated onto the medium containing (g/l): $NaNO_3$ 1 g, K_2HPO_4 1 g, KCl 1 g, $MgSO_4$ 0.5 g, yeast extract 0.5 g, glucose 1 g, carboxymethylcellulose (CMC) 5 g, and agar 15 g. The plates were incubated at 25°C for 5–8 days. At the end of the incubation, 0.2% (w/v) Congo Red solution was added to Petri dishes and kept at ambient temperature for 20 min. Then the Petri dishes were washed by adding 5 M NaCl solution to remove excess dye and kept at room temperature for another 30 min. Colonies with a light-yellow zone around the colony on a red background were evaluated as cellulase positive.

Protease activity. Protease activity was studied with modified method of Carrim et al. (2006). Nutrient Agar containing 1% (g/l) skimmed milk powder was used to prepare a protease substrate. Milk powder (10 g/100 ml) was sterilized at 110°C for 5 minutes, cooled to 45°C, and added to a basal medium in aseptic conditions. Strains inoculated onto the above medium were kept for two or three days at 25°C. A transparent zone formation around the colonies indicated a protease activity.

Pectinase activity. Pectinase activity was determined according to the method of Kobayashi et al. (1999). The isolates were inoculated onto the medium containing (g/l): yeast extract 2 g, ammonium sulfate 2 g, Na_2HPO_4 6 g, KH_2PO_4 3 g, pectin 5 g, and agar 15 g. The plates were incubated at 25°C for three days. At the end of incubation, after adding 1% (w/v) cetyltrimethylammonium bromide (CTAB) solution, the Petri dishes were kept at room temperature for 10 min. Transparent zone formation around the colony indicated a pectinase activity.

Xylanase activity. Xylanase activity was studied with a modified method of Amore et al. (2015). The isolates were inoculated onto the medium containing (g/l): $NaNO_3$ 1 g, K_2HPO_4 1 g, KCl 1 g, $MgSO_4$ 0.5 g, yeast extract 0.5 g, glucose 1 g, agar 15 g, and xylan 5 g. After the isolates were inoculated onto the medium, they were incubated at 25°C for two or four days. At the end of the incubation, 0.1% (w/v) Congo Red solution was poured into the Petri dish and staining was performed for 20 min. To remove the excessive dye, 5 M NaCl solution was added to the Petri dishes and kept at room temperature for 30 min. A light-colored zone on a red background indicated a xylanase activity.

Genotypic characterization of the selected isolates. Based on enzyme activities determined using solid selective media, 16 isolates were selected for diagnosis processes, giving successful and different EI values. The selected strains were identified by the 16S rRNA gene amplicon sequencing. DNA isolation was performed by the modified method of Govindarajan et al. (2007), and the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the genomic DNA as a template and universal bacterial primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') (Frank et al. 2008). A 50 µl reaction mixture contained 2.5 U Taq polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 0.3 mM dNTPs, 25 mM $MgCl_2$, 20 pmol of each primer, 5 µl of 10 x reaction buffer (Thermo Fisher Scientific), and 20 ng of template DNA. The step-up PCR procedure included denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min. Amplification products were electrophoresed on a 1.5% agarose gel in 1 × TBE buffer.

The 16S rRNA gene amplicon sequencing was performed by the Sentebiolab Biotechnology Company (Turkey) using the Miseq (Illumina) next-generation sequencing platform. The sequences obtained were analyzed using the database on the website (https://www.ezbiocloud.net), and then the sequences were logged in to the GenBank site and accessed "GenBank accession" numbers (Table II). The phylogenetic tree was created by the GGDC web server at http://ggdc.dsmz.de using the phylogenomic data line DSMZ (German Collection of Microorganisms and Cell Cultures GmbH) adapted to single genes (Meier-Kolthoff et al. 2013). Multiple sequence alignment was done with the "MUSCLE" (Edgar 2004), and the phylogenetic tree was created using the Maximum Likelihood method (Stamatakis 2014).

Statistical analysis. All enzyme measurement experiments were performed in four replicates, and each measurement on Petri dishes was repeated twice. The Statistical Analysis System (SAS version 9.4 SAS, Cary, NC) was used to analyze the data. General linear model (GLM) analysis was used to determine differences between the averages of the groups, and Duncan's multiple comparison test was used to determine differences between the groups. P values < 0.05 were considered statistically different.

Results and Discussion

In this study, a total of 128 endophyte bacteria isolated from various cultivated and wild grain plants (Poaceae family) were used. For all the isolates, the EI of each enzyme activity is given in Table SI. Since

Table II
Identification of strains according to the results of sequence analysis using the EzBioCloud database and GenBank accession numbers.

Code of the isolates	Top-hit reference species	Top-hit reference strain	Similarity (%)	Coverage (%)	GenBank Accession Numbers
G119Y1T	*Bacillus toyonensis*	BCT-7112	100.00	70.10	MW752891
G118S2T	*Pseudomonas congelans*	DSM 14939	100.00	100.0	MW752990
G117Y1T	*Streptococcus thermophilus*	ATCC 19258	94.58	89.30	MW774413
G116K1T	*Micrococcus luteus*	NCTC 2665	99.58	100.00	MW755305
G113Y3	*Bacillus halotolerans*	ATCC 25096	99.93	100.00	MW753050
G107Y2	*Curtobacterium flaccumfaciens*	LMG 3645	100.00	100.00	MW753051
G105Y1	*Bacillus subtilis* subsp. *inaquosorum*	KCTC 13429	99.92	84.50	MW753052
G105S1	*Bacillus idriensis*	SMC 4352-2	99.58	100.00	MW753132
G100Y1	*Paenibacillus nuruki*	TI45-13ar	99.25	100.00	MW753131
G90Y2	*Paenibacillus tundrae*	A10b	99.84	83.30	MW753134
G90S1	*Curtobacterium flaccumfaciens*	LMG 3645	100.00	100.00	MW757038
G88K1	*Pseudomonas orientalis*	CFML 96-170	99.62	89.20	MW753212
G83S3	*Paenibacillus seodonensis*	DCT19	99.23	88.00	MW753225
G80K3	*Paenibacillus xylanexedens*	B22a	99.80	100.00	MW753226
G70K2	*Paenibacillus xylanexedens*	B22a	99.80	100.00	MW753223
G42K2	*Chryseobacterium luteum*	DSM 18605	99.44	100.00	MW753224

endophytic bacteria offer a relatively new source of genes, enzymes, and secondary metabolites, we aimed to investigate several biotechnologically important extracellular enzymes of endophytic origin. By this purpose, endophytic bacteria isolated from Van province, Turkey, were evaluated for the presence of hydrolytic enzymes, including cellulases, xylanases, pectinases, amylases, proteases, and lipases (Fig. 1). They successfully demonstrated a variety of enzyme activities. It was revealed that lipases, proteases, amylases, cellulases, pectinases, and xylanases were produced with relative frequencies of 74.2%, 65.6% and 55.4%, 32%, 21.8%, and 7.8%, respectively (Fig. 2).

After the enzyme activity measurements were completed, 16 isolates revealing relatively high EI value for at least one enzyme tested were selected to perform a phylogenetic affiliation based on the 16S rRNA gene amplicon sequencing analysis. Also, among these selected strains, one producing none of the enzymes was selected for the identification (Table I).

The 16S rRNA gene amplicon sequencing of 16 isolates was successfully achieved. The ~ 1,500 bp 16S rRNA gene contains nine variable regions (V1–V9) in a highly conserved order. Since next-generation sequencing platforms provide an appropriate read of full-length the 16S rRNA gene intragenomic variants, they provide a better taxonomic resolution at species or strain level (Johnson et al. 2019). Illumina MiSeq method yielded full-length reading of the 16S rRNA gene amplicons for almost all strains. The lowest 16S rRNA gene reading length belongs to the strain G119Y1T with 70.1%, which nevertheless covers the V1–V5 regions (Johnson et al. 2019) (Table II). As a result of pairwise comparisons of the 16S rRNA gene sequences on EzBioCloud server, five *Paenibacillus* sp. (G100Y1, G90Y2, G83S3, G80K3, G70K2), four *Bacillus* sp. (G119Y1T, G105S1, G113Y3, G105Y1), two *Pseudomonas* sp. (G88K1, G118S2T), two *Curtobacterium* sp. (G107Y2, G90S1), one *Micrococcus* sp. (G116K1T), one *Streptococcus* sp. (G117Y1T), one *Chryseobacterium* sp. (G42K2) were identified (Table II).

Except for strain G117Y1T, the 16S rRNA gene amplicon sequencing results of all strains yielded 99–100% similarity (Table II). The 16S rRNA gene sequences alone may not be sufficient to identify a new species, but it can indicate that a new species is isolated (Tindall et al. 2010). The 94.58% similarity with G117Y1T is far below the threshold necessary to identify a new species (Stackebrandt and Goebel 1994; Stackebrandt and Ebers 2006), and, thus, this strain may represent a new species or even genus (Fig. 3). Noteworthy, strain G117Y1T gave positive results in terms of all enzymes except pectinase (Table I).

Different studies in the literature show that our identified strains belonging to seven different genera were endophytes (Verma et al. 2001; Rashid et al. 2012; Khan et al. 2017; Afzal et al. 2019). The different species of these genera produce high-potential bioactive compounds such as antimicrobials and enzymes to be used in the fields such as medicine and bioremediation, especially in agriculture (Doddamani and Ninnekar 2001; Schallmey et al. 2004; Lacava et al. 2007;

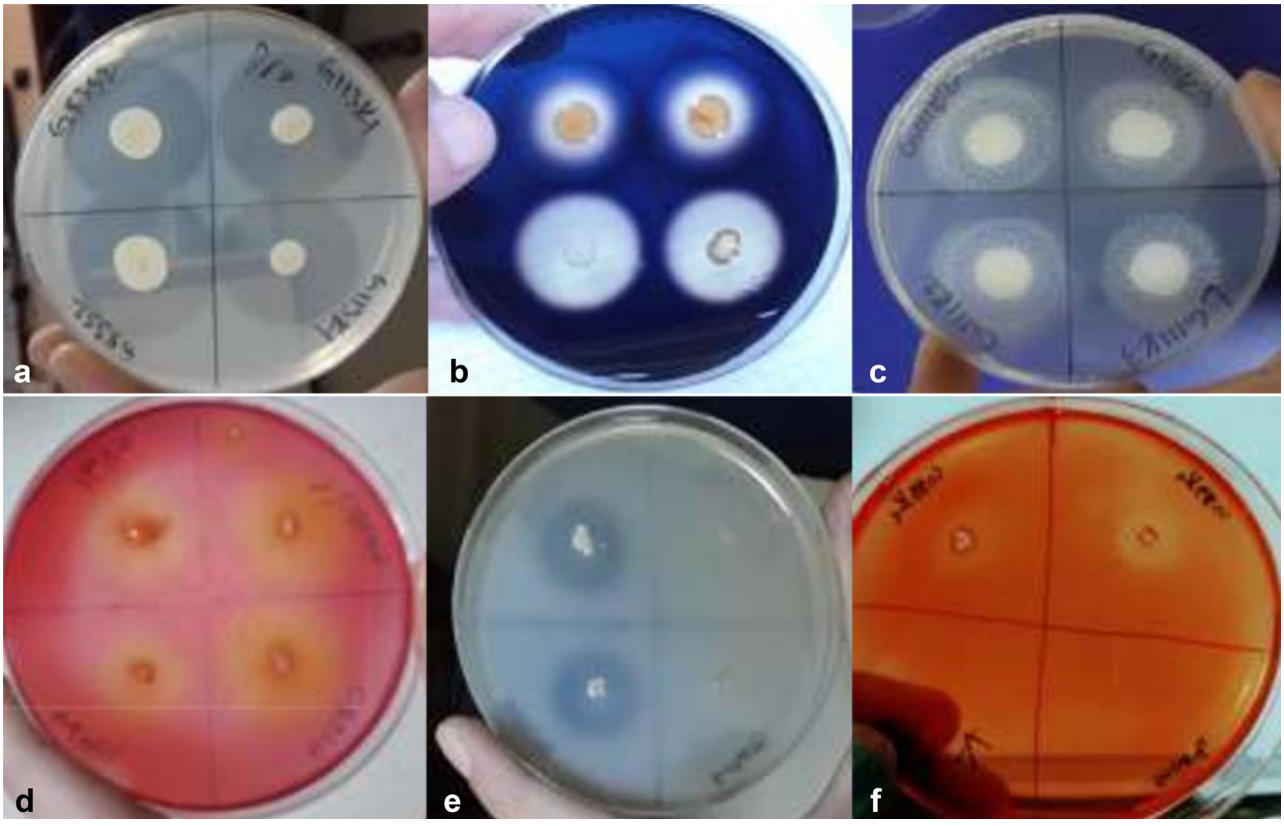

Fig. 1. Petri dishes with colonies surrounded by zones of extracellular enzymatic activity; a) protease, b) amylase, c) lipase, d) cellulase, e) pectinase, f) xylanase.

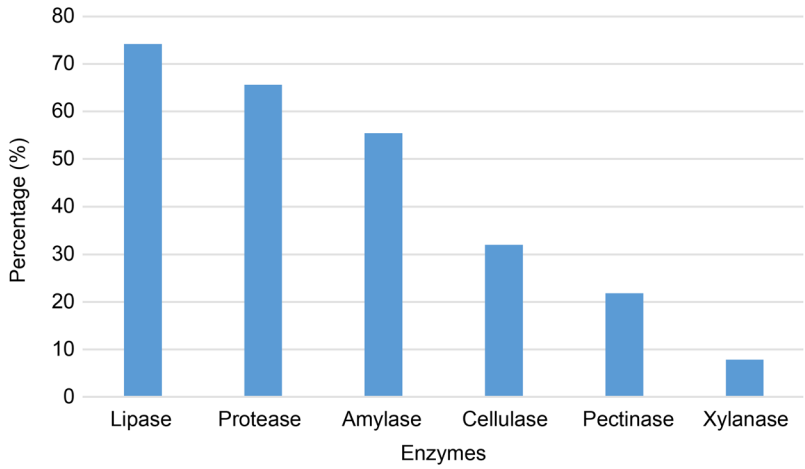

Fig. 2. Relative frequency (%) of strains (from a total of 128) producing individual hydrolytic enzymes.

Grady et al. 2016; Roy et al. 2018). Although the number of strains that we identified molecularly comprise a small cluster within all 128 isolates, they could reveal the diversity and support the literature data.

Carrim et al. (2006) presented the enzymatic activity of endophytic bacteria ranking as follows: protease (60%), amylase (60%), and lipase (40%). They did not detect cellulase and pectinase activities. Jalgaonwala and Mahajan (2011) detected 50% cellulase-positive endophytic bacteria in their study. On the other hand, our results revealed a high number of bacterial isolates with cellulase, lipase, and protease activities. Also, we have found a significant number of pectinase-positive isolates (Fig. 2). Despite the relatively limited number of studies, the percentage of endophyte bacteria with the positive scores for each of these enzymes varied due to the high species diversity.

Among the identified strains, *Bacillus* spp. (*B. toyonensis*, *B. halotolerans*, *B. subtilis* subsp. *inaquosorum*) except *B. idriensis* showed especially high cellulase

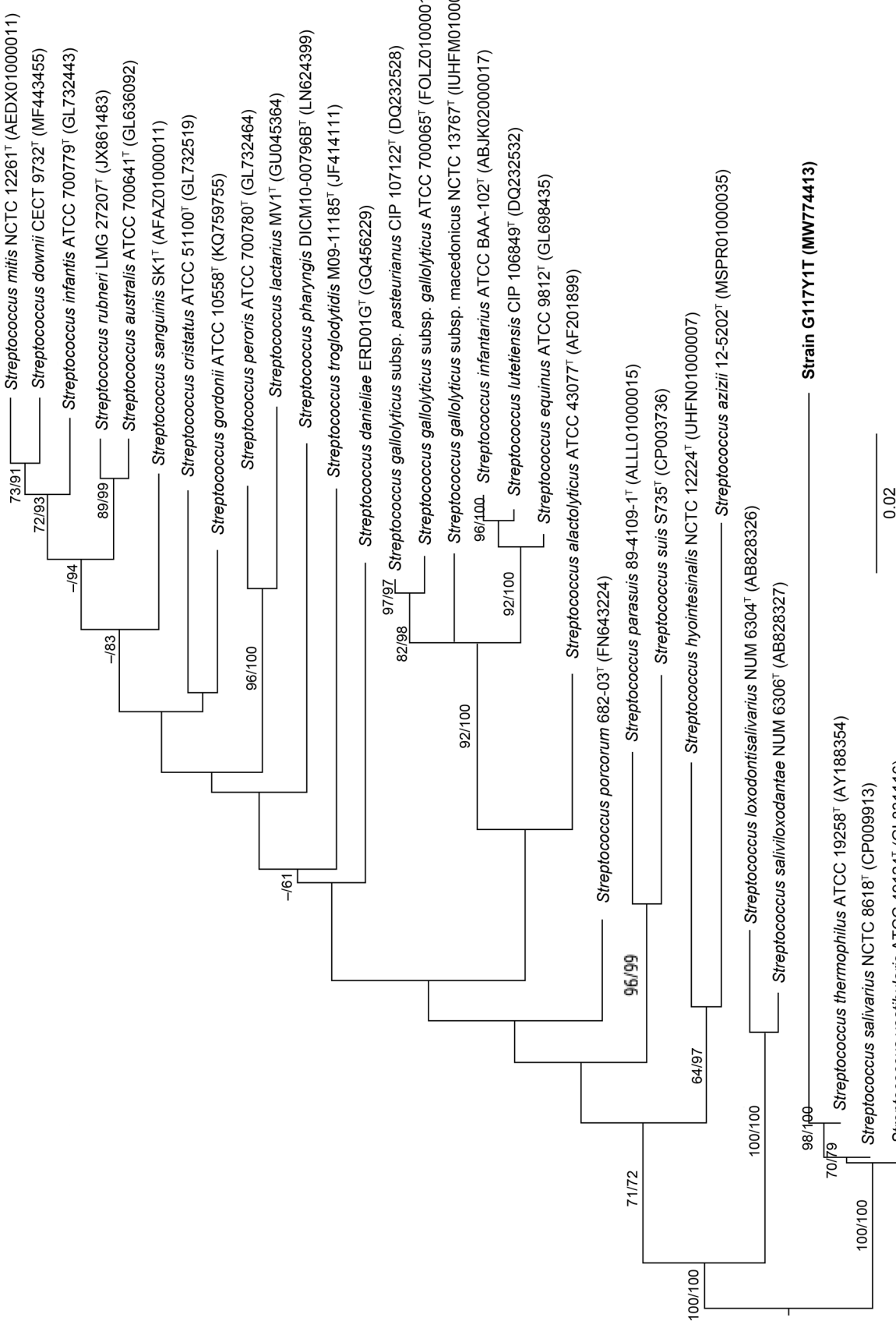

Fig. 3. The phylogenetic tree constructed by the maximum likelihood method and rooted by midpoint-rooting. Branches are scaled in terms of the expected number of nucleotide substitutions per site. Numbers at nodes indicate the bootstrap support values larger than 60% from maximum likelihood (left) and maximum parsimony (right) bootstrapping. GenBank accession numbers are given in brackets.

activity among six tested enzymes (Tables I and II). The strain G105S, which, in contrast to other strains, did not produce the above enzymes, was closely related to *B. idriensis* (99.58%) (Table II). However, *B. idriensis* that possessed protease, cellulase, and pectinase activities, was isolated as an endophyte in the study conducted by Afzal et al. (2017). *Pseudomonas* spp. (*P. congelans*, *P. orientalis)* were the main xylanase producers among identified strains. In general, the number of strains demonstrating xylanase activity was relatively low. For this reason, these strains belonging to the genus *Pseudomonas* are valuable as xylanase enzyme producers. Xylanases produced by bacteria (*Bacillus* spp., *Pseudomonas* spp., *Streptomyces* spp.) are efficient in a broad pH and temperature range. Therefore, they are very useful in different industries reciprocally (Burlacu et al. 2016). Among the isolates we described, *Paenibacillus* spp. was observed as the most productive group of lipases and cellulases. *Paenbacillus* species are known to produce different hydrolytic enzymes (Sakiyama et al. 2001; El-Deeb et al. 2013). Cho et al. (2008) isolated two cellulose hydrolase genes (*cel5A* and *cel5B*) from endophytic *Paenibacillus polymyxa*. The strain belonging to *Streptococcus*, *Micrococcus*, *Curtobacterium*, and *Chryseobacterium* showed high activity of proteases, lipases, and xylanases. Generally, in this study, Gram-positive bacteria displayed broader hydrolytic enzyme potential than Gram-negative bacteria. Published data revealed that endophyte diversity varies according to different territories, plants, and even different plant tissues (Akinsanya et al. 2015).

Although this study was carried out in line with the biotechnological perspective, extracellular enzymes should also be evaluated and discussed in terms of the relationship between endophyte bacteria and the plant hosts. For example, different levels of cellulases and pectinases were reported to be important in endophytic diazotrophic bacteria during plant cells colonization (Verma et al. 2001). Considering that the plant pathogen bacteria also synthesize the enzymes that break down the cell wall, more information about the expression and regulation of these enzymes in both groups could be crucial to understand and distinguish between these two groups of bacteria.

In this study, a potentiality of endophytic bacteria isolated from several grain plants (Poaceae family) in Van province, Turkey, to produce biotechnologically important enzymes, was revealed for the first time. Endophyte bacteria are rich sources of enzymes and new secondary metabolites for many industries due to their high species diversity and adaptation to different environments. Therefore, investigation of these isolates not only in terms of extracellular enzymes but also in terms of specific and industrially important secondary metabolites should be among the future.

ORCID
Bilgin Taskin https://orcid.org/0000-0002-9772-7438

Acknowledgments
This work was supported by the Scientific Research Project Units of Van Yuzuncu Yil University, Turkey (Project number: FYL-2018-7557). Also we sincerely thank Assist. Prof. Dr. Ahmet Akkopru, Van Yuzuncu Yil University, Turkey for his support.

Authors' contributions
BT designed the research. BT and GD conducted experiments, analyzed data, wrote and revised the manuscript. Both authors read and approved the manuscript.

Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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