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Research article

Identification of endophytic actinobacteria from Jerusalem artichoke and examination of inulinase gene and enzyme properties

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Abstract

Endophytic actinobacteria isolated from *Helianthus tuberosus* L. (Jerusalem artichoke) demonstrate great potency in the production of biological function and compounds, including exo-inulinase activity. Exo-inulinase is of interest to the beverage industry, as this enzyme hydrolyzes inulin stored within the Jerusalem artichoke tuber into fructose syrup in a single step. This study aimed to identify and investigate the exo-inulinase-producing endophytic actinobacteria from four varieties of Jerusalem artichoke (HEL65, JA89, CNS2867 and JA102×JA89) in root, leaf and stem samples. In total, 78 isolates of endophytic actinobacteria were classified into 24 different groups based on morphology and then identified based on the 16S rRNA gene into 5 genera: *Streptomyces* (64.1%), *Nocardia* (28.2%), *Actinosynnema* (3.9%), *Kribbella* (2.6%) and *Kineococcus* (1.3%). The results of inulinase screening using polymerase chain reaction with degenerated primers showed the specific band at 400 bp of *Streptomyces gancidicus* EAH-R3 which conserved the protein of the exo-inulinase gene and had inulinase activity of 125 U/mL. This inulinase was confirmed to be an exo-inulinase by checking the end product of inulin hydrolysis using thin layer chromatography. The crude enzyme of *S. gancidicus* EAH-R3 had an optimum temperature and pH of 60°C and 6.0, respectively. Moreover, the crude inulinase was thermostable at 80°C for 2 hr with a relative activity of 94%. *S. gancidicus* EAH-R3, a new isolate of endophytic actinobacteria isolated from the plant with high inulin content, is a candidate for fructose production using inulin hydrolysis.

Introduction

Jerusalem artichoke (*Helianthus tuberosus* L.) is a perennial C-3 warm-season plant which is native to North America (Slimestad et al., 2009). This plant is of increasing interest recently as it has a number of advantageous characteristics: it has a high growth rate, can be extensively cultivated for improving poor soil, has good tolerance to frost and drought, has low fertilizer requirements and has resistance to plant diseases and pests (Slimestad et al., 2009). For the last two decades, Jerusalem artichoke has been one of the important sources for inulin production along with chicory root (Judprasong et al., 2011). The inulin of the Jerusalem artichoke tuber is considered important in functional foods (such as prebiotic from inulin and oligofructose), bioethanol production, fructose syrup in the food industry, bioactive compounds, green or ensiled forage and in the production processes of various chemicals such as butyric acid, lactic acid and citric acid

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(Van den Ende et al., 2004). Moreover, inulin can be completely hydrolyzed to its monomer becoming fructose syrup, which is widely used as sweetener instead of sucrose and glucose in pharmaceuticals as well as the food and beverage industries (Kaur and Gupta, 2002). As fructose is a sweetener used in food industry, it has more beneficial effects for diabetic or obese patients because the fructose metabolism bypasses the glucose metabolic pathway and does not require insulin in this pathway (Millo and Werman, 2000).

Microbial inulinases, identified to be an important class of industrial enzymes, have attracted increasing attention in recent years (Kango and Jain, 2011). Inulinases can be produced by yeasts, bacteria and fungi, where fungi are more studied than any other microbe (Kango and Jain, 2011). Inulinases are fructofuranosyl hydrolase that catalyzes on the β-2,1 linkage of inulin, producing inulo-oligosaccharides, with fructose as the main product and small amounts of glucose. They can be divided into endo-inulinase and exo-inulinase. The endo-inulinase enzyme hydrolyzes the internal linkage of the inulin to inulotriose, inulotetrose and inulopentaose as the main products but they lack invertase activity (Slimestad et al., 2009). The function of the exo-inulinase enzyme is to remove terminal fructose residues from the non-reducing end of the inulin chain to become fructose and glucose (Slimestad et al., 2009). Exo-inulinases (EC.3.2.1.80) or 2,1 β-D-fructopyranoside fructohydrolase is specific inulin hydrolysis and sequentially releases terminal fructose molecule from the non-reducing end of the inulin molecule attacking the terminal β-(2→1)-fructofuranosidic bonds (Chen et al., 2009). The conventional production approach yields fructose from starch using serial enzymatic methods involving α-amylase, amyloglucosidase and glucose isomerase (Gao et al., 2007). However, the best procedure for fructose syrup production is one-step enzymatic hydrolysis of inulin from microbial inulinase yielding 95% pure fructose (Chi et al., 2009). Consequently, exo-inulinase is the best choice to use for production of ultra-high-fructose syrup from inulin and inulin-containing materials in one easy step.

Many genes of exo- and endo-inulinase are obtained from yeasts, filamentous fungi and bacteria, with yeasts such as *Pichia* sp., *Cryptococcus aureus* and *Kluyveromyces marxianus* being inulinase producers with higher potential than filamentous fungi and bacteria (Laloux et al., 1991). The exo-inulinase (*INU1*) gene was found in *Kluyverumyces marxianus* (Laloux et al., 1991) and represented the first gene cloning in *Pichia guilliermondii* strain 1 (Gong et al., 2008) and *C. aureus* G7a (Sheng et al., 2008); moreover, *KcINU1* exo-inulinase gene was found in *Kluyveromyces cicerisporus* CBS4857 (Wen et al., 2003). In addition, exo-inulinase genes has been identified in various bacteria in the genera *Pseudomonas*, *Bacillus*, *Geobacillus* and *Lactobacillus*, with the *inu2* exo-inulinase gene in *Pseudomonas mucidolens* isolated from soil (Kwon et al., 2000), the *inu* gene from *Bacillus polymyxa* MGL21 encoded protein of 485 amino acids (Kwon et al., 2003), the thermophilic bacterium *Geobacillus stearothermophilus* KP1289 consisting of the *inuA* gene encoding 493 amino acids (Tsujimoto et al., 2003) and finally, *Lactobacillus casei* IAM1045 exhibited the *levH1* gene that actively degrades inulin (Kuzuwa et al., 2012). Generally, the lengths of the known inulinase genes are in the range 1,542–1,670 bp, being cloned from marine yeast and terrestrial yeast, resulting in deduced molecular masses of 51–59 kDa (Chi et al., 2009). The lengths of the extracellular inulinase genes from bacteria are in the range 1,482–1,536 bp (Chi et al., 2009). All the inulinase enzymes contain the consensus conserved motifs R-D-P-K-V-F-W-H and W-M-N-D-P-N-G and in addition, the consensus motif (WMNDPNGL) acts as a nucleophile and another consensus conserved motif (RDPKVF) has a key role in catalytic activity (Chi et al., 2009). The conserved region of the exo-inulinase gene can be detected using polymerase chain reaction (PCR), with corresponding degenerate primers offering a rapid and efficient method (Laloux et al., 1991).

Endophytic actinobacteria have attracted attention among ecologists, chemists, agronomists, taxonomists and evolutionary biologists, because of their potential for beneficial biological activity, such as antitumor, antibiotic and disinfectant agents, as enzymes and as plant growth promoters (Berdy, 2005). Such potential has led to an interest in exploring inulinase for its ability to hydrolyze inulin, particularly from the Jerusalem artichoke tuber. Presently, there are no reports of endophytic actinobacteria being isolated from Jerusalem artichoke. The purposes of this study were the isolation of endophytic actinobacteria from four varieties of Jerusalem artichoke in order to identify the species of actinobacteria, and to search for the exo-inulinase gene from the isolated actinobacteria using PCR with degenerate primers. This knowledge may lead to further study of the inulinases of actinobacteria to improve understanding of gene expression and the function of the enzyme inside its plant host, and for biotechnology applications.

Materials and Methods

Plants sampling

Four varieties of Jerusalem artichoke (HEL65, JA89, CNS2867 and JA102×JA89) were collected for bacterial isolation from the Field Crop Research Station of Khon Kaen University in Khon Kaen province, Thailand. Whole plants in the age range 25–30 d were collected in different seasons. The samples were transported to the laboratory for immediate isolation.

Isolation of endophytic actinobacteria

Plant materials were washed with tap water and dried at room temperature, followed by a five-step surface sterilization as described by Qin et al. (2009). The water used in the last rinse was analyzed to check the effectiveness of the sterilization procedure by spreading on a plate and observing bacterial growth. The endophytic actinobacteria were isolated from surface-sterilized roots, stems and leaves of Jerusalem artichokes. The surface-sterilized samples (1 g) were ground in a sterilized grinding bowl and 10 mL of saline solution added. The suspensions were spread over the surface of solid media designed for cultivation of endophytic actinobacteria. Several media were used for endophytic actinobacteria isolation to support endophytic

actinobacteria growth as shown in Table 1. The culture plates were incubated at 30°C for 7–14 d. Endophytic actinobacteria colonies that appeared on the culture plates were purified on isolated media. The purified actinobacteria were grown on ISP2 medium for working culture.

Morphological characterization of endophytic actinobacteria

All isolates were tentatively grouped, based on their morphology and cultural characteristics on agar plates. The spore suspension of endophytic actinobacteria was prepared by growing bacteria on ISP2 slant for 7 d at 30°C. Five microliters of spore suspension were dropped on ISP3 medium and incubated at 30°C for 7 d. The macro morphological characteristics observed were colony color, distinctive reverse colony, spore mass color and diffusible pigment. The reverse of substrate and aerial mycelia were determined under microscopy (100×).

Molecular characterization using 16S rRNA gene

Genomic DNA was extracted from the endophytic actinobacteria in a method modified from Chassy and Giuffrida (1980). The mycelia were filtered through Whatman filter paper No.4 and 100–200 mg of mycelia were collected in 1.5 mL microtubes. Two hundred microliters of 0.01 M Tris-HCl buffer (pH 8.2) were added and then ground using a grinding bar that fitted into the bottom of the microtube. The ground mycelia were added with 400 µL PEG (24% w/v) and inverted 5–10 times before adding with 200 µL lysozyme solution (20 mg lysozyme in 1.6 mL of 0.02 M Tris-HCl buffer pH 8.0), incubated at 37°C for 1 hr and centrifuged at 12,000 revolutions per minute (rpm) for 5 min. The pellets were suspended in 400 µL of 0.01 M Tris-HCl (pH 8.2) mixed by pipetting. The cell suspension was added with 50 µL of 10% SDS, inverted 20–30 times and then left at room temperature for 5 min. After that, 400 µL of 0.3 M sodium acetate and 600 µL of (1:1) phenol: chloroform were added, and the solution inverted 20 times before centrifugation at 12,000 rpm for 10 min. The aqueous phase was transferred into new microtubes, with 1 volume of isopropanol added, inverted 20 times and then kept at -20°C for 20 min before centrifugation at 13,000 rpm for 15 min. The DNA pellets were precipitated and washed again using 70% ethanol. The DNA pellets were dried and suspended in TE buffer (pH 8.0) and then kept at -20°C until use.

The endophytic actinobacteria were subjected to 16S rRNA gene sequence analysis for species identification. The genomic DNA of each isolate was amplified using a pair of the universal primers: 27F (5´-AAGGTTTGATCMTGGCTCAG-3´) and 1492R (5´-TACGGYTACCTTGTTACGACTT-3´) of the 16S rRNA gene. The PCR mixture (50 µL) contained at final concentration of 1× reaction buffer, 0.2 mM each of deoxynucleoside triphosphates, 2 mM $MgCl_2$, 0.5 µM each primer, 1 U of *Tag* DNA polymerase (Thermo Fisher Scientific; USA) and 1 ng of template DNA. The PCR reaction was performed under the following conditions: initial denaturation at 94°C for 3 min; 34 cycles of 94°C for 1 min; 55°C for 30 s; 72°C for 1.5 min; and final extension at 72°C for 10 min using a Thermal cycler (FlexCycler^2; Germany). The PCR products were visualized on 1% agarose gel stained with ethidium bromide under UV light transilluminator (Quantum ST4; Vilber Lourmat; Germany). The PCR products were purified by GeneJET PCR Purification Kit (Thermo Fisher Scientific; USA) before being sent for analysis by a DNA sequencing service (First BASE laboratory in Malaysia). The 16S rRNA sequences obtained were compared with the GenBank database using the BLASTn search program NCBI BLAST (https://blast.ncbi.nlm.nih.gov) and the secondary database, EzBioCloud (https://www.ezbiocloud.net). A phylogenetic tree was constructed using the neighbor-joining method with the MEGA7 program (Kumar et al., 2016). The reliability of branching was performed using the bootstrap method for 1,000 bootstrap replications.

Table 1 Media used for isolation and characterization of endophytic actinobacteria

Media	Composition per liter	References
ISP*2 (yeast extract-malt extract agar)	4 g yeast extract, 10 g malt extract, 4 g dextrose	Shirling and Gottlieb, 1966
ISP*5 (glycerol asparagine agar)	1 g L-asparagine, 10 g glycerol, 1 g K_2HPO_4, 1 ml trace salts (0.001 g $FeSO_4 \cdot 7H_2O$, 0.001 g $MnCl_2 \cdot 4H_2O$, 0.001 g $ZnCl_2 \cdot 7H_2O$)	Shirling and Gottlieb, 1966
TWYE (tap water-yeast extract agar)	0.25 g yeast extract, 0.5 g K_2HPO_4, tap water 1 L	Crawford et al., 1993
GYM (glucose yeast-malt extract)	4 g yeast extract, 10 g D-glucose, 4 g malt extract	Whitman et al., 2012
YECD (yeast extract-casein hydrolysate agar)	0.5 g yeast extract, 0.5 g D-glucose, 2 g K_2HPO_4	Whitman et al., 2012
GSA (Gause's synthesis agar)	20 g soluble starch, 5 g NaCl, 1 g KNO_3, 5 g $K_2HPO_4 \cdot 3H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 4 mg $FeSO_4 \cdot 7H_2O$	Whitman et al., 2012
ZSPE (Zhang' starch plant extract agar)	5 g soluble starch, 1 g KNO_3, 10 mL Jerusalem artichoke plant extract	This study
ISPS (International Streptomyces Project - Sucrose)	4 g yeast extract, 10 g malt extract, 4 g sucrose	This study

*ISP = International Streptomyces Project medium.

Determination of exo-inulinase gene using degenerated primers

Degenerated primers of the exo-inulinase gene were designed from the amino acid conserved sequence of the exo-inulinase gene from several bacteria in the NCBI GenBank database. The conserved amino acid sequences were used for forward degenerated primer design as "WMNDPNG", and "DFRDPKV" was used for reverse degenerated primer design. The nucleotide sequences of conserved amino acid regions were compared and designed for degenerated primers, called EI_F (5'-TGGATGAAYGAYCCVAACG-3') and EI_R (5'-ACTTTNGGATCSCGRAAATC-3') for forward and reverse primers, respectively.

Every endophytic actinobacteria isolate was analyzed for the exo-inulinase gene using PCR with the degenerated primers (EI_F and EI_R in this study). The PCR reaction mixture (50 µL) contained at final concentration of 1× reaction buffer, 0.2 mM each of deoxynucleoside triphosphate, 2 mM $MgCl_2$, 0.5 µM each primer, 1 U of *Tag* DNA polymerase (Thermo Fisher Scientific; USA) and 1 ng of template DNA. The PCR was performed under the following conditions: initial denaturation at 94°C for 3 min; 34 cycles of denaturation at 94°C for 1 min; annealing at 48°C for 1 min; extension at 72°C for 1 min; and a final extension at 72°C for 10 min. Five microliters of PCR products were mixed with 2 µL of (1/500×) fluorescent dye (UltraPower DNA Stain; China) before separating using 1% agarose gel electrophoresis. The PCR bands were visualized under a UV light transilluminator (Quantum ST4; Vilber Lourmat; Germany). The predicted size of the PCR product was about 400–600 base pairs.

DNA cloning and sequencing

The PCR products from exo-inulinase screening with a length of about 400–600 bp were verified for the inulinase gene by sequencing. In brief, the PCR products were purified using a GeneJET PCR Purification Kit (Thermo Scientific; USA) and then inserted into a TA cloning vector following the RBC TA Cloning Vector Kit protocol. After ligation, the recombinant DNA was transformed into *Escherichia coli* TOP10 competent cells using electroporation (Electro Cell Manipulator® ECM 630; USA). The electroporation conditions were: voltage at 2,500 V, resistance 200 Ω, capacitance 25 µF in 2 mm BTX Disposable Cuvettes Plus (BTX; USA). One ml of Super Optimal broth with Catabolite repression (SOC medium) was immediately added after electroporation and incubated at 37°C and 150 rpm for 1 hr. The cell suspension was spread on Luria Bertani (LB) plates containing 50 µg/mL of ampicillin containing 20 µL of 50 mg/ml X-gal and 100 µL of 100 mM IPTG. A blue-white colony appeared on the LB plates after incubation at 37°C for 16 hr and the white colony was selected for plasmid extraction using the alkali method following Sambrook et al. (1989). The recombinant plasmid was purified before determination of the sequence at the First BASE laboratory (Malaysia). The DNA sequences of the inulinase gene from the recombinant clone were translated into amino acid sequences and identified using blastp in the NCBI database.

Assay of enzyme activity

The endophytic actinobacteria carrying the inulinase gene were analyzed for enzyme activity by culturing in ISP2 broth and incubation at 30°C and 150 rpm for 7 d. The supernatant was filtered through a membrane to collect the crude enzyme. Inulinase activity was detected by measuring the amount of fructose released after inulin hydrolysis by exo-inulinase. One hundred microliters of crude enzyme were added into 900 µL of 1% inulin (from chicory; Sigma; Japan) in 0.1 M phosphate (pH 6.0) and incubated at 60°C for 1 hr before the enzyme was stopped by boiling for 15 min. The control used the inactive crude enzyme (by boiling for 15 min) in the reaction. The free fructose content was analyzed following Saengkanuk et al. (2011). Briefly, 150 µL of crude enzyme reaction was mixed into 5 mL of 20 mM citrate buffer (pH 6.0) and added with 100 µL of 10 mM sodium periodate, and 4.6 mL of water. The reaction was mixed using a vertex mixer and allowed to stand for 5 min. After that, 150 µL of 100 mM potassium iodide was added and mixed using the vortex mixer. After 5 min, solution absorbance was measured at 350 nm using a UV-Vis spectrophotometer (Optizen 3220UV; Korea). The concentration of free fructose was deduced from the calibration curve of standard fructose. One unit of enzyme was defined as the amount of exo-inulinase that relieved 1 µM of fructose per minute.

The optimum pH was investigated by incubating the enzyme with 1% inulin in different buffers at pH values of 4–9. The buffers were 0.1 M citrate buffer (pH 4–5), 0.1 M phosphate buffer (pH 6–8) and 0.1 M glycine/NaOH (pH 9). The inulinase activity was determined as described above. The optimum temperature was tested at 40–80°C for reaction incubation of inulinase activity. For temperature stability, the enzyme was pre-incubated at 4°C, 35°C, 60°C and 80°C for 2 hr. Residual activity was measured and the relative inulinase activity calculated.

Determination of exo-inulinase hydrolysis using thin layer chromatography

The end products of the inulin hydrolyzed by the exo-inulinase were detected using thin layer chromatography (TLC) method. Eight hundred microliters of 1% inulin (Sigma; Japan) in 0.1 M phosphate buffer (pH 6.0) was added to 200 µL of crude enzyme and then incubated at 37°C in a water bath for 48 hr. A 0.5 µl sample solution was spotted on Silica gel 60 F_{254} (Merck; USA) compared with three standard sugars at 0.1%, namely raffinose (trisaccharide), sucrose (disaccharide) and fructose (monosaccharide) and 1% inulin. The TLC was run in the mobile phase composed of ethyl acetate:acetic acid:methanol:water (12:3:3:2 volume per volume) according to Suárez-González et al. (2014). The TLC was left to dry at room temperature for 10 min and then developed using thymol-sulfuric spray (0.5 g of thymol in 95 mL of 95% ethanol and 5 mL of 96% sulfuric acid), followed by heating at 120°C for 10 min. The spots that appeared were pink in color.

Results

Endophytic actinobacteria isolated from Jerusalem artichoke

Jerusalem artichoke is an interesting plant due to high level of inulin storage in its tubers (Slimestad et al., 2009). The endophytic actinobacteria living inside Jerusalem artichoke is important in the study for exo-inulinase-producing bacteria. In total, 78 endophytic actinobacteria were isolated from the four varieties of plant based on eight media (Table 1) to receive endophytes containing the inulin hydrolysis enzyme in different species. Analysis discovered that the colony morphology and numbers for ZSPE and ISPS were not different from ISP2, which previously displayed good growth for the Actinomycetes group. Table 2 shows that endophytic actinobacteria were abundant in the JA102×JA89 variety. The majority of endophytic actinobacteria were found in the roots (65.4%), compared to the leaves (20.5%) and stem (14.1%). The colonies were greatest in *Streptomyces*, while there were less than half as many in *Nocardia*; few other genera were found.

The 78 isolates of endophytic actinobacteria were classified into 24 groups using their different morphology on ISP3 medium (that showed growth from poor to abundant), the color of the substrate and aerial mycelia, melanoid pigment production and on spore structure (Table 3). In terms of morphology, microscopic observation found coccoid (*Kineococcus*), rod-straight (*Actinosynnema*), fragment (*Kribbella*), fragment-mycelia (*Nocardia*) and branched mycelia (*Streptomyces*). On the other hand, 24 endophytic actinobacteria were identified using 16S rRNA sequence into 4 main groups, 5 genera and 24 species and shown in a phylogenetic tree (Fig. 1). The first group, EAH-S1 isolate, exhibited an orange-pigment colony which did not produce aerial mycelia on medium, nor coccoid under microscopy. The result of 16S rRNA sequencing indicated that this isolate was *Kineococcus radiotolerans* with identity of 99% (Table 3). In the second group, two isolates had close lineage to the genus *Actinosynnema* and the genus *Nocardia* (Fig. 1). The results of 16S rRNA sequencing delineated two isolates from each other as *Actinobacteria*, namely *Actinosynnema murium* (EAJ-R11) with 98% identity and *Nocardia beijingensis* (EAJ-R5) with 100% identity, with their colony morphologies being yellow- and brown-pigmented on agar, respectively. The third group was another *Actinobacteria*, being the EAC-L3 isolate that had white substrate mycelia on the ISP3 medium. This information was confirmed using 16S rRNA sequencing which indicated that EAC-L3 was *Kribbella shirazensis* with identity of 99%. The fourth group was the genus *Streptomyces,* which had different types of spore morphology such as straight, flexuous, hooked, primitive spirals, opened loops, opened spirals and closed spirals (Table 3). This group was divided into three sub-groups (Fig. 1): group IV (i) was non-spore *Streptomyces* (EAJJ-S1 identified as *Streptomyces guanduensis* with 98 % identity); group IV (ii) consisted of a variety of spore morphologies such as opened loops, hooked, primitive spirals and opened spirals, composed of: EAH-R3, EAJJ-S3, EAH-L2, EAJJ-R7, EAC-R1, EAJ-R1, EAJJ-R11 and EAJJ-R6. The last group, group IV (iii) consisted of closed spirals, straight, flexuous and flexuous to straight spores consisting of: EAC-S2, EAH-R7, EAC-S4, EAH-R1, EAC-L7, EAJ-R13, EAH-R6, EAH-R9, EAH-R5, EAJJ-R2 and EAC-S1. The results of 16S rRNA sequencing identified the last two sub-groups of group IV to the genus *Streptomyces* with a range identity of 98–100%. All 24 sequences were submitted into the NCBI database and given accession number as shown in Table 3.

Table 2 Distribution of endophytic actinobacteria in different varieties of Jerusalem artichoke isolated from different plant material (root, stem, leaf) and numerous genera obtained from the current study

Plant sample/ Bacterial type	Isolate number	Isolate number (%)
Plant variety		
HEL65	12	15.4
CNS2867	13	16.7
JA89	24	30.8
JA102×JA89	29	37.2
Plant materials		
Root	51	65.4
Stem	11	14.1
Leaf	16	20.5
Type of endophytic actinobacteria		
Streptomyces	50	64.1
Nocardia	22	28.2
Kribbella	2	2.6
Kineococcus	1	1.3
Actinosynnema	3	3.9

Inulinase gene screening in endophytic actinobacteria

The endophytic actinobacteria of the 78 isolates were screened for inulinase-production capacity using PCR with degenerated primers designed from conserved amino acid sequences. The results of PCR screening consisted of 18 isolates that generated positive PCR products with lengths in the range 200–500 bp (Fig. 2). Almost all isolates showed a single band at about 400 bp. Three isolates (*S. gancidicus* EAH-R3, *S. albogriseolus* EAJ-R1 and *N. beijingensis* EAJ-R5) had PCR products of 400 bp, 350 bp and 200 bp, respectively. They were chosen to verify the gene by cloning into TA cloning vectors and sequencing. The DNA sequences of the three clones indicated that only the *S. gancidicus* EAH-R3 clone had an inulinase fragment. This was confirmed using the translated protein sequence blast in the NCBI protein database. The protein sequence from *S. gancidicus* EAH-R3 had a putative conserved domain of G32_β_fructosidase which is a glycoside hydrolase of family 32 (GH32) with the WMNDPNG motif in GH32 (Fig. 3). The results confirmed that the 400 bp of the PCR product from *S. gancidicus* EAH-R3 encoding 131 amino acids was the inulinase enzyme.

Table 3 Morphological and molecular characterization of endophytic actinobacteria

Group	Isolate* (Accession number)	Species	Number of members#	Macro-observation on ISP3 medium					Micro-observation	
				Growth	Color of mycelia		Soluble pigment	Water drop	Spore chain	Spore diameter (μm)
					Substrate	Arial				
I	EAH-S1 (KY908428)	*Kineococcus radiotolerans*	1	Poor	Orange	None	None	None	None	0.5
II	EAJ-R11 (KY908441)	*Actinosynnema mirum*	3	Abundant	Yellow	Grayish white	Yellow	None	Straight	0.5–2
	EAJ-R5 (KY908439)	*Nocardia beijingensis*	22	Abundant	Brown	White	Light Brown	None	None	1
III	EAC-L3 (KY908436)	*Kribbella shirazensis*	2	Moderate	White	White	None	None	None	1
IV(i)	EAJJ-S1 (KY908450)	*Streptomyces guanduensis*	1	Poor	None	Brown red	None	None	None	None
IV(ii)	EAH-R3 (KY908423)	*Streptomyces gancidicus*	3	Abundant	White	Grey	None	Build	Closed spirals	1–1.5
	EAJJ-S3 (KY908451)	*Streptomyces flavomacrosporus*	1	Abundant	Cream	Dark gray	None	Build	Primitive spirals	1.5
	EAH-L2 (KY908429)	*Streptomyces massasporeus*	4	Abundant	Light pink	gray	Light Pink	Build	Opened spirals	1–2
	EAJJ-R7 (KY908447)	*Streptomyces rochei*	1	Abundant	Brown	Dark grey	None	None	Flexuous	0.25
	EAC-R1 (KY908430)	*Streptomyces geysiriensis*	2	Abundant	Yellow	Grey	Yellow	Build	Primitive sprials	1
	EAJ-R1 (KY908438)	*Streptomyces albogriseolus*	1	Moderate	White	Grey	None	Build	Flexuous	1
	EAJJ-R11 (KY908448)	Streptomyces coeruleorubidus	1	Abundant	Green cream	Grayish green	None	Build	Sprials	1
	EAJJ-R6 (KY908446)	*Streptomyces bellus*	2	Abundant	Green	Grayish green	None	Build	Hooks	0.75
IV(iii)	EAC-S2 (KY908432)	*Streptomyces vellosus*	1	Abundant	White	Grey	None	Build	Closed spirals	0.5
	EAH-R7 (KY908426)	*Streptomyces* sp.	2	Abundant	White	Grey	None	Build	Closed spirals	1
	EAC-S4 (KY908434)	*Streptomyces hokutonensis*	3	Abundant	Orange yellow	White	None	None	Straight	1–1.5
	EAH-R1 (KY908422)	*Streptomyces cellostaticus*	2	Abundant	Light blue	Grey	None	Build	Closed spirals	1
	EAC-L7 (KY908437)	*Streptomyces phaeopurpureus*	3	Moderate	Cream	White	None	None	Straight	1–2
	EAJ-R13 (KY908442)	*Streptomyces heliomycini*	3	Abundant	Cream	Cream white	None	None	Straight	1–2
	EAH-R6 (KY908425)	*Streptomyces panayensis*	6	Abundant	White	Greyish white	None	Build	Straight	1–2
	EAH-R9 (KY908427)	*Streptomyces bungoensis*	2	Abundant	Yellow	Greyish white	Yellow	None	Flexuous	0.5–1
	EAH-R5 (KY908424)	*Streptomyces griseoruber*	2	Abundant	White	Grey	None	None	Flexuous to straight	1–2
	EAJJ-R2 (KY908444)	*Streptomyces coelicolor*	2	Abundant	Cream	Grey	Light brown	None	Flexuous to straight	0.5–1
	EAC-S1 (KY908431)	*Streptomyces caeruleatus*	8	Abundant	Orange brown	White	Orange	None	Straight	1

EA = endophytic actinobacteria; H = HEL65; C = CNS2867; JJ = JA102×JA89; J = JA89; R = root; S = stem; L = leaf.

* numbers are serial isolates of one type of samples.

members in each isolate were classified using morphological characterization.

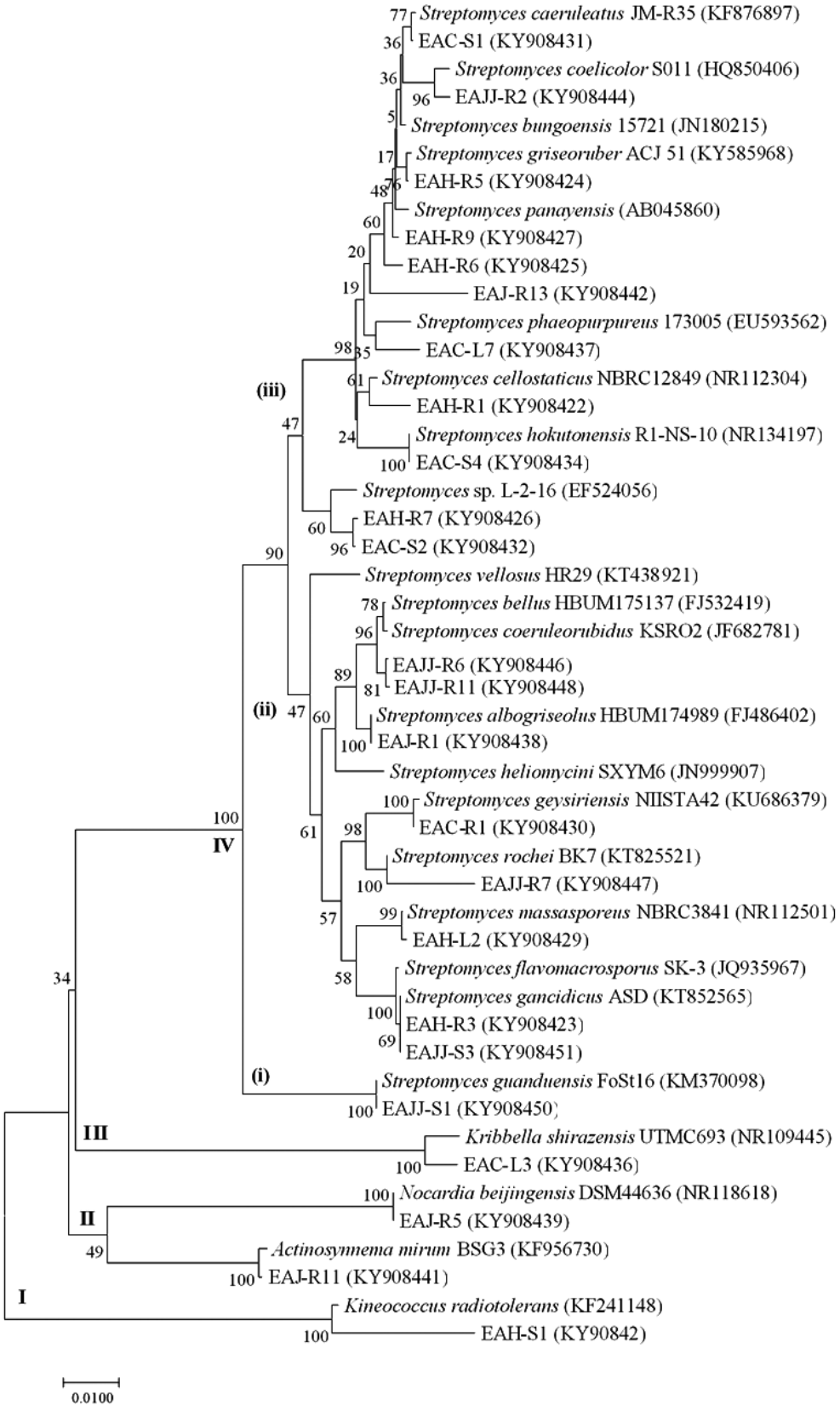

Fig. 1 Phylogenetic tree of endophytic actinobacteria isolated from four varieties of Jerusalem artichoke using neighbor joining analysis of the 16S rRNA sequence, consisting of four groups (I–IV) of genera and three sub-groups (i–iii) of species in *Streptomyces*, where branch length and scale bar equal the number of nucleotide substitutions per site that changed among taxa

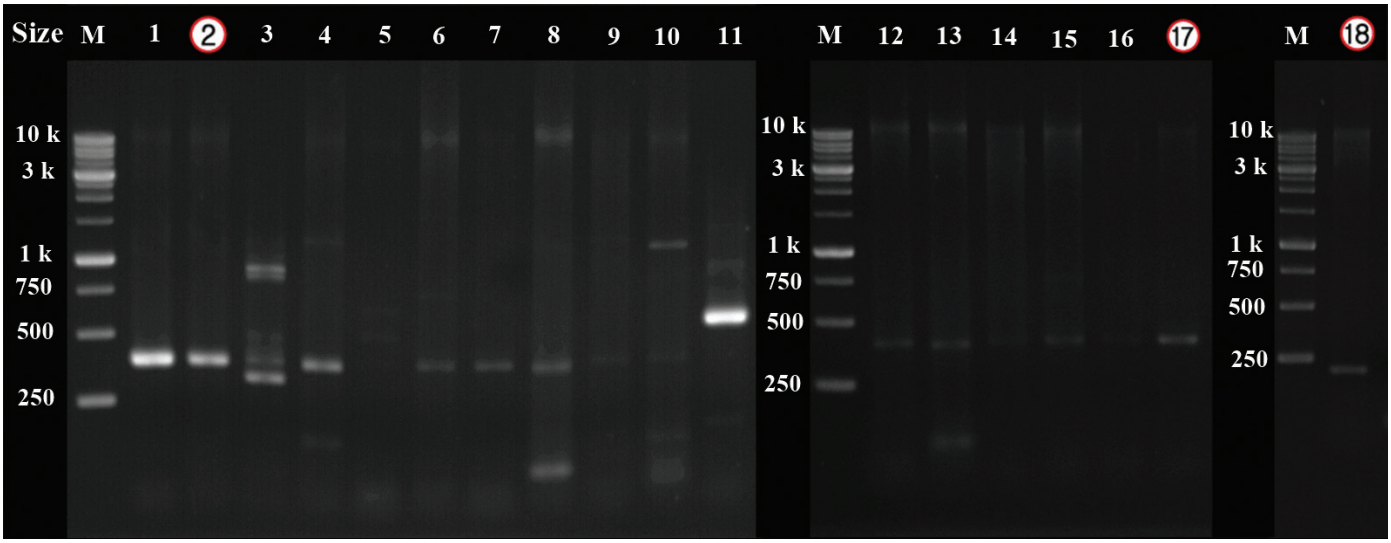

Fig. 2 Positive bands of polymerase chain reaction product screened from endophytic actinobacteria using EI_F and EI_R primers, where M = 1 kb DNA ladder RTU (Gendered, Taiwan); 1 = *S. cellostaticus* EAH-R1; 2 = *S. gancidicus* EAH-R3; 3 = *S. griseoruber* EAH-R5; 4 = *S. massasporeus* EAH-L2; 5 = *S. caeruleatus* EAJ-R20; 6 = *S. coelicolor* EAJJ-R2; 7 = *S. gancidicus* EAJJ-R9; 8 = *S. griseoruber* EAJJ-R13; 9 = *S. heliomyces* EAJJ-R16; 10 = *A. mirum* EAJJ-S5; 11 = *S. geysiriensis* EAC-S3; 12 = *K. radiotolerans* EAH-S1; 13= *K. shirazensis* EAC-L3; 14 = *S. caeruleatus* EAJ-R16; 15 = *S. cellostaticus* EAJJ-R5; 16 = *S. geysiriensis* EAC-R1; 17 = *S. albogriseolus* EAJ-R1; 18 = *N. beijingensis* EAJ-R5

A.

(EI_F primer)
TGGATGAAYGAYCCVAACG
TGGATGAATGACCCGAACGGCCTCGTGTACTACAAGGGCGAATACCACCTCTTCTACCAGT
ACAACCCGAATGGCAACTCCTGGGGCGACATGTCCTGGGGGCACGCGGTGAGCACGGACC
TCGTGCACTGGAAGCAGCTGCCGCTGGCCCTGTCGTACGACGACAAGGAGATGGTCTTCTC
CGGCAGCGCGGTGGTCGACTGGAACAACACCACCGGGTTCGGCACGAAGAAGAACCCGCC
CATGGTGGCGGTCTACACCAGCTACTCCAAGTCCACGGGCACGCAGGCCCAGGCCCTCGCC
TACAGCACCGACCGCGGCCGCACCTGGACCAAGTACCAGGGCAATCCCGTCATCGACATC
GGCTCCAGGGATTTCCGGGATCCAAAAGTA
GATTTYCGSGATCCNAAAGT (Reverse complementary EI_R primer)
ACTTTNGGATCSCGRAAATC (EI_R primer)

B.

WMNDPNG (EI_F primer)
WMNDPNGLVYYKGEYHLFYQYNPNGNSWGDMSWGHAVSTDLVHWKQLPLALSYDDKEM
VFSGSAVVDWNNTTGFGTKKNPPMVAVYTSYSKSTGTQAQALAYSTDRGRTWTKYQGNPVI
DIGSR**DFRDPKV**
DFRDPKV (EI_R primer)

Fig. 3 *S. gancidicus* EAH-R3 fragment from degenerated primers (EI_F and EI_R): (A) nucleotide sequence; (B) amino acids sequence (matched with GH32_β_ fructosidase in Protein BLAST), where bold and underline indicate primer positions

Inulinase activity of S. gancidicus EAH-R3

The inulinase activity of *S. gancidicus* EAH-R3 was 125.10 U/mL hydrolysis of inulin. The optimum pH of this enzyme was pH 6; it worked well in acidic conditions, whereas in contrast, its activity was very low for an alkaline pH (Fig. 4A). The ideal temperature for the inulinase was 60°C (Fig. 4B) and the enzyme had 80–98% relative activity after 180 min at 4°C, 35°C and 60°C (Fig. 4C). However, the enzyme still retained more than 60% relative activity at 80°C.

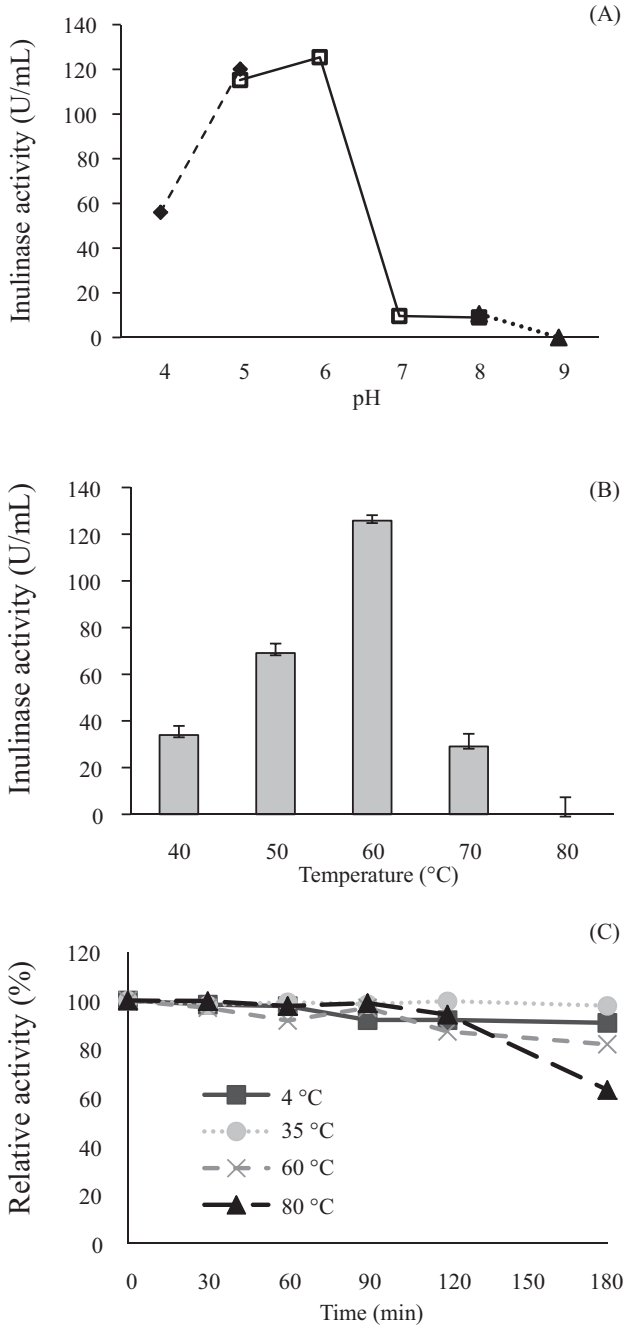

Fig. 4 Optima for inulinase enzyme from *S. gancidicus* EAH-R3 isolated from Jerusalem artichoke: (A) pH; (B) temperature, where error bars indicate ±SD; and (C) enzyme stability at different temperature

Additionally, the inulinase of *S. gancidicus* EAH-R3 was incubated with 1% inulin for 48 hr followed by inulinase hydrolysis using TLC. The results indicated that the end-product after inulin hydrolysis was fructose; therefore, the inulinase of *S. gancidicus* EAH-R3 was verified as exo-inulinase (Fig. 5). This result confirmed *S. gancidicus* EAH-R3 as an exo-inulinase-producing strain.

Discussion

Endophytic actinobacteria were isolated from Jerusalem artichoke and characterized at the genus and species levels using colony and mycelial morphology; moreover, the relationships among them were analyzed using 16S rRNA sequencing. Table 3 shows the numbers of endophytic actinobacteria isolated from different parts of the plant varieties, with five genera identified, for which 64.1% of the total endophytic actinobacteria belonged to *Streptomyces* strains. Additionally, *Nocardia* and *Streptomyces* strains were detected in the four plant varieties. Endophytic actinobacteria were found predominantly in the plant root, rather than in the leaf or stem. The endophytic actinobacteria of Jerusalem artichoke have not been previously isolated and their ecology studied; therefore, this work reported the genera of the endophytic actinobacteria from *Helianthus tuberosus* L. (Jerusalem artichoke) consisting of: *Streptomyces*, *Nocardia*, *Kineococcus*, *Kribbella* and *Actinosynnema*. Additionally, *N. beijingensis* (28.2%) was observed in the largest numbers in all varieties. This finding was consistent with the results of Nimnoi et al.

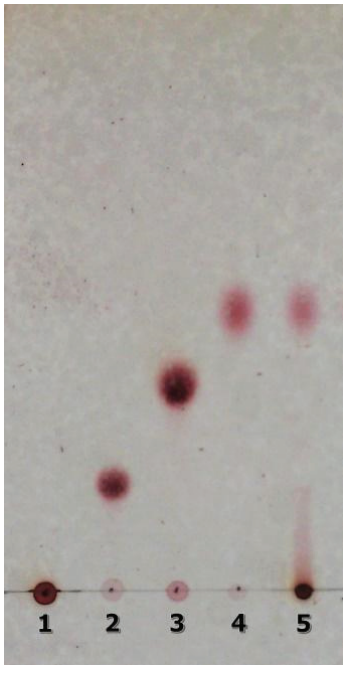

Fig. 5 Inulin hydrolysis of exo-inulinase from *S. gancidicus* EAH-R3 by thin layer chromatography, where 1 = 1% inulin; 2 = 0.1% raffinose (trisaccharide); 3 = 0.1% sucrose (disaccharide); 4 = 0.1% fructose (monosaccharide); 5 = end product after hydrolyzation of inulin by exo-inulinase

(2010) who mentioned that *Nocardia* was most frequently found in *Aquilaria crassa* collected from Nakhon Nayok province, Thailand. Gangwar et al. (2014) investigated endophytic actinomycetes in 30 banana trees with a predominance of *Streptomyces* (68.3%) followed by *Nocardia* in 11.7% by isolation. It is likely that the distribution of endophytic actinomycetes shows the same features as in the plant host, including for Jerusalem artichoke.

Of the four varieties of plant studied for endophytic actinobacteria, only HEL65 had the EAH-S1 isolate which was close to *K. radiotolerans.* In addition, the CNS2867 variety had the EAC-S2 isolate which was identified as *S. vellosus*. The EAJ-R1 isolate was most related to *S. albogriseolus* in the JA89 variety. The JA102×JA89 variety was obtained from *S. rochei* EAJJ-R7, *S. coeruleorubidus* EAJJ-R11, *S. guanduensis* EAJJ-S1 and *S. flavomacrosporus* EAJJ-S3 isolates. However, some endophytic actinobacteria were widespread in two varieties, such as, *S. caeruleatus* EAC-S1, and *K. shirazensis* EAC-L3 and *A. mirum* EAJ-R11 which were found in the CNS2867 and JA89 varieties. This implied that the endophytes were able to colonize plants without a specific host; however, they preferred to live inside some hosts independently and these included different varieties. The distribution of endophytes depends on their environment, such as the soil, as they can grow and spread outside the plant host during their life cycle.

Endophytes have a close correlation with their host plants. Therefore, the endophytic actinobacteria that inhabit the Jerusalem artichoke may be involved in inulin accumulation by producing inulin synthesis or inulin hydrolysis enzymes. Plants accumulating inulin have sucrose:sucrose 1-fructosyl transferase (1-SST) for inulin synthesis and fructan 1-exohydrolase (1-FEH) for inulin degradation (Van den Ende et al., 2004). The current work investigated endophytic actinobacteria for the inulinase gene, using degenerated primers. Approximately 10% (8 isolates of 78 total isolates) showed levels of positive products containing 400 bp. This indicated that few endophytic actinobacteria isolated from high inulin plant carry the inulinase gene. Thus, the inulinases of endophytes may be helper enzymes for plants under stress conditions and not specifically for inulin accumulation, as the FEH functions as an energy supply and increases osmotic pressure to protect plants under stress conditions. However, the inulinase of endophytic actinobacteria had high activity, with 125 U/mL in *S. gancidicus* EAH-R3. Information on inulinase biosynthesis of actinobacteria is scarce and is mainly characterized in bacteria and expressed in *E. coli* (Liu et al., 2013). Moreover, there have been four exo-inulinases reported in *Streptomyces* consisting of *Streptomyces* sp. CP01 (Laowklom et al., 2012), exo-inulinase of *Streptomyces* sp. GNDU 1 (Gill et al., 2003), exo-inulinase of *Streptomyces* sp. ALKC4 isolated from the rhizosphere soil of chicory (Sharma and Gill, 2007) and exo-inulinase of *Streptomyces grisenus* isolated from soil (Tohamy, 2006). The current work concluded that *Streptomyces gancidicus* EAH-R3 is a new endophytic actinobacterium of Jerusalem artichoke which produces extracellular exo-inulinase.

The exo-inulinase of *S. gancidicus* EAH-R3 had hydrolysis ability at a high temperature (60°C) and in weak acids (pH 5–6); in addition, the enzyme was stable at high temperatures. It is likely to have a long shelf life at low temperatures. This finding was in accord with Sharma and Gill (2007) who suggested that the inulinase produced from *Streptomyces* sp. ALKC4 had optimum activity at 60ºC and pH 6.0 with good stability at this temperature. In contrast *S. grisenus* (Tohamy, 2006) had an optimum pH range of 7–7.5. Furthermore, the *S. gancidicus* EAH-R3 enzyme was more thermostable than exo-inulinase from *Streptomyces* sp. ALKC4. *S. gancidicus* EAH-R3 had good stability at 80ºC for 3 hr, retaining activity of about 60%, while the heat-stable exo-inulinase of *Streptomyces* sp. ALKC4 had high activity at 70ºC for 3 hr with retained activity of about 50%. The results demonstrated that the endophytic actinobacteria from plants with a high inulin content contain the exo-inulinase enzyme which has potential properties suitable for industrial application with only mild conditions require din processing. Furthermore, the exo-inulinase gene of *S. gancidicus* EAH-R3 should be characterized for gene expression to further study the relationships between plant-endophytic actinobacteria.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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