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ORIGINAL ARTICLE

Isolation and Cloning of an Inulinase Gene from an Endophytic Bacteria

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Abstract: In this study, an endophytic bacterium was isolated from *Black Solenostemon scutellarioides* plant and was named as A5. A simple and rapid method is described for the detection of inulinase-producing bacterial endophyte on agar plate using Remazol Brilliant Blue-inulin as substrate. The inulinase activity of A5 bacteria isolate was detected by clear zone surroundings the colonies. The inulinase gene obtained from A5 bacterium was amplified by the polymerase chain reaction. Degenerate primers were used specifically designed to pick up the inulinase gene. pGEM-T Easy and *E. coli* JM109 were used as cloning vector and host strain, respectively. The results showed that the partial sequence of inulinase gene consisted of 449bp. From the comparison with partial amino acid sequence of inulinase, the deduced polypeptide showed high sequence similarity to *Paenibaccillus polymyxa* and *Geobacillus stearothermophilus* exo-inulinases.

Key words: Endophytic bacteria , inulinase, oligosaccharides, RBB-inulin, gene cloning.

Introduction

Endophytes, by definition, are organisms including bacteria that live in close association with living plant tissues [10, 13]. Endophytic bacteria have been found in virtually every plant studied and can form a range of different relationships including symbiotic, mutualistic, commensalistic and trophobiotic. Endophytic bacteria can promote plant growth and can act as biocontrol agents. Endophytes can also be beneficial to their host by producing a range of natural products that could be harnessed for potential use in medicine, agriculture or industry [11]. Some endophytic bacteria have been report to be inulin degraders to produce fructose or other oligosaccharides [4, 9, 10].

Inulin is a widespread polyfructan with linear chains of β-(2→1)-linked fructose residues attached to a terminal sucrose molecule. Inulin occurs as a reserve carbohydrate in plants such as chicory, Jerusalem artichoke, asparagus and dahlia. Depolymerization of inulin involves the action of enzymes namely, inulinase. They can be divided into exo-inulinases and endo-inulinase [3]. Endo-inulinase hydrolyzes inulin through endo-reaction (cleavage from inside) producing fructo-oligosaccharides. Exo-inulinase, as the name implies, are exo-actings, which split off terminal fructose units successively from non-reducing end of the inulin molecule.

Inulinase have been isolated from microorganisms including bacteria. The genes encoding for inulinase also have been cloned from *Aspergillus niger* [8,16], *A. awamori* [1], *Geobacillus stearothermophilus* KP1289 [15], *Arthrobacter* sp. [5] and *Kluyveromyces marxianus* [7]. Inulinase from microorganisms has potential applications in reducing production costs and improving syrup quality. In this

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study, we described the isolation of inulinase gene from endophytic bacteria, isolated from *Black Solenostemon scutellarioides*. The inulinase activity was assayed using dye-coupled techniques.

Degenerate upstream and downstream primers were used specifically designed to pick up the inulinase gene. Here we also demonstrate that the inulinase partial gene sequence was successfully cloned in *E. coli*.

Materials and Methods

Chemicals:

All the chemicals were purchased from Sigma Chemical Co., Missouri USA, unless otherwise stated.

Isolation of Endophytic Bacteria:

At first the plant of *Black Solenostemon scutellarioides* or locally name hati-hati hitam was collected at Institute of Bioscience, Universiti Putra Malaysia, Selangor, Malaysia. The plants were washed with running tap water. Stem (1 cm) of the plant were separated and subjected to surface sterilization treatment to eliminate the contaminating microflora. Sterilization was performed in sterile 6-well tissue culture plate (Costar) by immersing in 75% (v/v) ethanol for 1 min, 0.5% of sodium hypochlorite solution for 5 min and 75% (v/v) ethanol for 1 min. After that, the plant stem were washed in autoclaved distilled water gently and were soaked in autoclaved tissue paper [14]. The plant tissues were aseptically placed in sterile petri dishes and allowed to air dry in laminar flow. Segments were halved and teased throughout its length with a fine needle to expose the plant tissue. Each segment was placed onto Nutrient Agar (NA) media. The plates with plant tissues were incubated at 28 ºC for 1 to 3 weeks to allow endophytic bacteria to grow.

Colonies were picked and then were purified by single-colony streaking on NA plates. The bacteria isolate was designated as A5.

Screening of Inulin Degrading Endophytes: Dye-labeled Substrate Synthesis:

Briefly, 5.0 g inulin and 0.625 g Remazol Brilliant Blue (RBB) was dissolved separately in two flasks, each containing 62.5 ml of distilled water and then mixed together. The mixture was then heated at 50 ºC for 1 hour and at different times, equal portions of Na_2SO_4 (total amount 12.5 g) were added stirring the mixture vigorously. Then, 6.25 ml of Na_3PO_4 solution were added to the reaction mixture and maintained at 50 ºC for another hour. The resulting mixture was centrifuged at 5,000 g (Sigma 3k30 B. Braun) for 25 min at 5 ºC and the supernatant was discarded. The precipitate was resuspended in 6.25 ml of distilled water. Then, 62.0 ml of 95% ethanol were added and the suspension was centrifuged as described above. This procedure was repeated 5 times to obtain colourless supernatants. Afterwards, the precipitate was resuspended in ethanol and dried at room temperature.

Detection of Rbb-inulin Degrading A5 Bacteria Isolate:

The isolated of A5 endophytic bacteria were inoculated onto RBB-inulin with the following compositions: 1.8 g agar, 1.2 g RBB-inulin, 0.24 g NH_4NO_3, 0.06 g KH_2PO_4, 0.06 g $MgSO_4$. $7H_2O$, 0.06 g KCl, 0.001 g $FeSO_4$. $7H_2O$ and 0.024 g yeast extract and per 120 ml distilled water. The RBB-inulin with actively growing endophytic bacteria A5 isolate was then incubated at 28 ºC for 24 hours. Positive results showed clear zone around the bacteria colony[2].

Isolation and Manipulation of Nucleic Acids of A5 Isolate:

An A5 yielding inulinase was originally isolated from *Black Solenostemon scutellarioides.* The *bacterium* A5 was grown in Nutrient Broth (NB). Genomic DNA of this organism was extracted by using the Wizard® Genomic DNA Purification (Promega, Madison, USA). The concentration of the extracted genomic DNA was estimated spectrophotometrically using a UV/Visible spectrophotometer.

PCR Amplification and Dna Sequencing of Inulinase Gene":

Genomic DNA was amplified by polymerase chain reaction (PCR) using the degenerate upstream primers InuF2: 5'-TGGATGAAYGAYCCIAAYGGICTIG-3' and the downstream primers InuR2: 5'-RAAIACYTTIGGRTCYCTRAARTC-3' specifically designed to pick up the inulinase gene. The primers were designed according to the highly conserved domains of the bacterial inulinase protein. The PCR reaction system consisted of 2.5 µl PCR buffer (10X), 1.5 µl $MgCl_2$ (25mM), 0.5 µl dNTP mix (10 mM each of dATP, dCTP, dGTP and dTTP), 1.0 µl of InuF2 Forward and InuR2 Reverse (15 µM) primers respectively, 0.25 µl Taq DNA polymerase (5 U/µl), 1.0 µl template DNA (1 µg/µl) and distilled water made up to a final volume of 25 µl. Amplification was done in a PCR Thermal Cycler (MJ research, Inc. PTC-100) with the following

parameters: 1 cycle at 94 ºC for 2 minutes, 35 amplification cycles of 1 min at 94 ºC, 1 min at 45 ºC, 1 min at 72 ºC and an additional extension period of 3 min at 72 ºC. The PCR product was detected using gel electrophoresis in 1.2% (w/v) agarose. The electrophoresis was carried out at 80 volt for 45 min. Then, the PCR product was cut from the gel and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA).

Preparation of Competent E. coli JM109 Cells:

Competent *E. coli* cells were prepared by using the $CaCl_2$ method according to Sambrook et al. (12) with slight modification. *E. coli* cells of JM109 were plated on LB (1.0 g tryptone, 0.5 g yeast extract, in 100 ml distilled water) medium overnight. A single colony was inoculated into 50 ml S.O.C medium and incubated at 37 ºC for 3 to 4 hours with vigorous shaking until the absorbance (A_{660}nm) reached 0.4 to 0.8. The culture was chilled by storing the tube on ice for 10 minutes before centrifugation at 1,157 x g for 15 minutes at 4 ºC to recover the cells. After the media was discarded, the cell pellet was resuspended in 10 ml of ice-cooled 0.1 M $CaCl_2$ and stored on ice for 10 minutes at 4 ºC. The cells were recovered by centrifugation for 15 minutes at 4 ºC and the pellet was then resuspended in 2 ml of ice-cooled 0.1 M $CaCl_2$ solution. Aliquots of about 200 µl of the competent cells were dispensed into microcentrifuge tubes and can be used immediately. The ice-cooled sterile glycerol was added to the cells and stored at -80 ºC for future use.

Ligation and Transformation of the Gene into Plasmid:

The purified PCR product was ligated into pGEM®-T Easy Vector (Promega, Madison, USA) and transformed into *E. coli* JM109. Colonies from successful transformations were picked up and grown.

Clone Analysis by PCR:

From the LB/ampicillin/IPTG/X-Gal plates, single colony with transformed recombinant vector was picked and resuspended in 30 µl of the PCR mixture. The reaction mixture was incubated for 5 min, at 94 ºC to lyse the cells and inactivate nucleases. Amplification of 35 cycles through PCR with conditions as described above was performed and PCR products were visualized by agarose gel electrophoresis. The isolated plasmid DNA of the inulinase gene was then submitted for sequencing (First BASE Laboratories Sdn. Bhd). The sequences were compared with databases available at the National Center for Biotechnology Information (NCBI) using the BLAST program.

Results and Discussion

A5 Isolate and Inulin-degrading Endophyte:

An unidentified bacterial endophyte was successfully isolated from *Black Solenostemon scutellarioides* and was named as A5 in this study. The inulinase activity of A5 bacteria isolate was assayed using dye-coupled techniques [2]. Inulin covalently linked with RBB was prepared as a substrate. Microbial hydrolysis was detected in RBB-inulin agar plate by clear zone (Diameter of 1.2 cm) surroundings the colony of A5 bacteria isolate.

Inulinase Gene of Bacterium A5:

Genomic DNA isolated from endophytic *bacterium* A5 was used for PCR (Figure 1). A band indicating a fragment about 0.4 kb in size was shown by agarose gel electrophoresis (Figure 2). The band was cloned and sequenced after recovery and purification. The sequences producing significant alignments with *bacterium* A5 are presented in Table1.

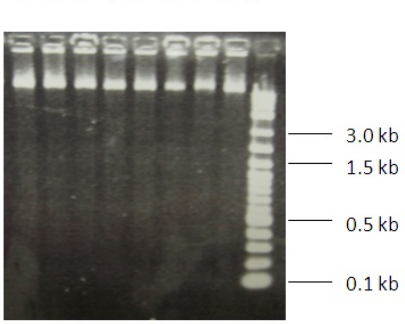

Fig. 1: DNA samples extracted from Bacterium A5. Lane 1, 2, 3, 4, 5, 6, 7, 8: Bacterium A5 DNA; Lane 9: 2-Log DNA Ladder (BioLabs).

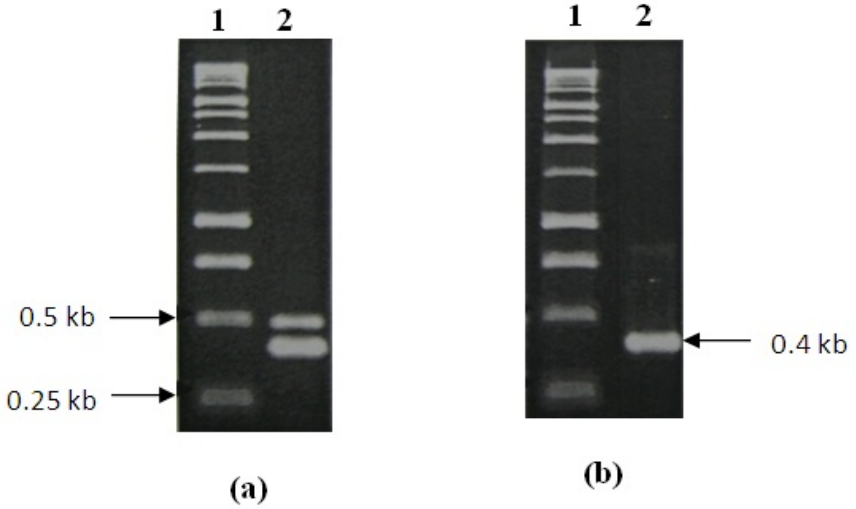

Fig. 2: (a) PCR amplification of inulinase gene (b) The purified PCR product of inulinase gene. Lane 1: 1 kb DNA ladder; Lane 2: PCR product of A5 as a template.

1	TGGATGAACG	ATCCGAATGG	GCTGGTCTAT	TTCGAGGGCG	AATATCATCT
51	GTTCTTCCAG	CATCATCCCG	CAGGCATGAC	AATGGGGGAT	ATGCACTGGG
101	GCCATGCCGT	CAGCAAGGAC	CTGATCCACT	GGGTGGAACT	GCCGATTGCG
151	CTTGCACCGG	ATGAGCTGGG	CATGATCTTT	TCCGGAAGTG	CTGTGGTGGA
201	CTGGAACAAT	ACGACGGGAT	TTTTCGGGGA	GAAACCGGGA	CTGGCAGCGG
251	TCTTTACACA	TCATCTGGAT	ATGCCGGAGG	GACAGCCTGC	GGTTCAGGTC
301	CAGAGTCTGG	CCTACAGCAA	TGACAAGGGC	AGAACCTGGA	CGAAATATGA
351	GGGAAATCCG	GTGATTACGC	ACGAGACCTT	CATTGATTTT	AGGGACCCCA
401	AAGTCAATCA	CTAGTGAATT	CGCGGCCGCC	TGCAGGTCGA	CCATATGGG

Fig. 3: Partial nucleotide sequence of inulinase gene from *Bacterium* A5.

1	WMNDPNG	LVYFEGE	YHLFFQH	HPAGMTM	GDMHWGH
51	AVSKDLI	HWVELPI	ALAPDEL	GMIFSGS	AVVDWNN
10	TTGFFGE	KPGLAAV	FTHHLDM	PEGQPAV	QVQSLAY
15	SNDKGRT	WTKYEGN	PVITHET	FIDFRDP	KV

Fig. 4: Partial amino acid sequence of inulinase gene from *Bacterium* A5.

Table 1: Sequences producing significant alignments with *bacterium* A5

Accession no.	Description	Total score	Query coverage	Identity
YP 003244249	Glycosyl hydrolase family 32 domain protein, *Geobacillus* sp. Y412MC10	217	100%	71%
AAL82575	exo-inulinase, *Paenibacillus polymyxa*	201	100%	68%
YP 003013913	Glycosyl hydrolase family 32 domain protein, *Paenibacillus* sp. JDR-2	199	100%	71%
BAC45010	exo-inulinase, *Geobacillus stearothermophilus*	196	100%	66%
AAF44125	exo-inulinase, Pseudomonas mucidolens	192	100%	66%

The results showed that the partial sequence of inulinase gene consisted of 449 bp (Figure 3). From the comparison with partial amino acid sequence (Figure 4) of inulinase, the deduced polypeptide showed 68% and 66% identities to that of *Paenibacillus polymyxa* [6] and *Geobacillus stearothermophilus* (15) exo-inulinases, respectively.

The endophytic *bacterium* A5 also showed significant degrees of identity to that of *Pseudomonas mucidolens* (66%) exo-inulinase (Table 1). These results suggest that 449 bp fragment of the inulinase partial gene sequence from endophytic *bacterium* A5 was successfully cloned in *E. coli*.

Conclusion

Successful amplification of inulinase partial gene sequence was achieved from endophytic *bacterium* A5. The amplified product was cloned into *E. coli*. This result will help to study the regulation of inulinase gene expression in the bacterial endophyte in future.

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