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Cloning, Sequencing and Analyzing of 16S rRNA Gene from Inulin Hydrolyzing Bacteria

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ABSTRACT

Gene of 16S rRNA is used for molecular identification of bacteria. This research aims to analyze the sequences of 16S rRNA gene from inulin hydrolyzing bacteria of UKG isolate. The 16S rRNA gene was isolated by PCR using BacF1 and UniB1 primers. The gene was cloned to pGEM-T *Easy* vector with *E.coli* TOP10F as host cell. Recombinant DNA was sequenced using T7 and SP6 primers. The size of the nucleotide base of recombinant DNA of sequencing result using SP6 primer was 1167 bp, whereas using T7 primer was 1218 bp. The both of the sequences overlapped 760 bp. The size of 16S rRNA gene in the sequences was found 1501 bp. Recombinant DNA that was restricted using *Eco*R1 showed 3 bands on agarose gel. They were 3000 bp, 800 bp, and 700 bp. The position of the *Eco*R1 palindrome sequence on the 16S rRNA gene was at the 832-837. The 16S rRNA gene also has been known by *Hind*III, *Bam*HI, *Bal*I, *Hae*III, and *Sma*I restriction enzymes.

Key Words: *inulin hydrolyzing bacteria, 16S rRNA gene, pGEM-T Easy, inulin.*

1. INTRODUCTION

Fructose is a compound that has a higher sweetness than sucrose and glucose [1]. Therefore, fructose is used as an important sweetener in food and beverage industry. Fructose can be produced from hydrolysis of starch using the α-amylase, amyloglucosidase and glucose isomerase enzymes [2]. Starch hydrolysis produced 42% fructose using the enzymes. Otherwise, inulin hydrolysis produced 98% fructose using inulinase [3]. Therefore, fructose production from inulin hydrolysis is more promising and has received tremendous attention in recent years.

Inulin is a natural polymer of carbohydrate groups. Inulin is composed of 2-60 fructose molecules that connected by β-(2-1) glycoside bonds and generally has a glucose terminal molecule that connected to fructose via α-(1-2) glycoside [4]. Inulin was applied widely in the food, beverage and pharmaceutical industries. Inulin is a prebiotic that classified as a food ingredient [2].

Inulin can be hydrolyzed to produce fructose and fructooligosaccharide using the inulinase that can be produced by microorganisms. Bacteria is one of a potential microorganisms that produce inulinase or levanase [5]. One of the sources of inulin hydrolyzing bacteria is bacteria from tuber of rhizosphere *Dahlia* sp. Inulin hydrolyzing bacteria can be identified using the 16S rRNA gene. The 16S rRNA gene is a type of RNA found in the prokaryotic ribosome. Gene of 16S rRNA is used for bacterial classification widely. The use of 16S rRNA gene as a classification of bacteria is more appropriate and trusted than other types of RNA gene [6].

The 16S rRNA gene is universal gene. The gene has a conserved region and few variable region. The variable regions are used as a differentiator of bacteria from one another [7], while the conserved region are used to design specific primers [8]. The use of 16S rRNA gene is also caused by the adequate size (about 1500 bp) [9]. The difference of 16S rRNA gene sequences can be used to determine the evolutionary relationship between bacteria organisms, archaebacteria, and eukaryotics [10]. Inulin hydrolyzing

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bacteria of UKG isolate had been screened by previous researchers [11]. This study aims to determine the size and analyze the 16S rRNA gene sequence from the UKG isolate.

2. MATERIALS AND METHODS

2.1 The Cultured Bacteria of UKG Isolate

The UKG isolate was cultured in the medium (g/L), 2.0 g $(NH_4)_2SO_4$, 14.0 g KH_2PO_4, 6.0 g $K_2HPO_4.3H_2O$, 0.2 g $MgSO_4.7H_2O$, 1.0 g of trisodium citrate, 10 g of inulin, and 20 g bacto agar [12]. The temperature of bacterial growth was 37ºC

1.2 Isolation of Genomic DNA from UKG Isolate

Isolation and purification of bacterial genomic DNA was carried out according to procedure in the Wizard Genomic DNA Purificarion Kit Promega. Cultured bacteria of UKG isolate (18 hours, exponential phase) was put in 1.5 mL microtube and centrifuged at 12.000 rpm, 4ºC for 2 minutes. Pellet was added 480 µL EDTA 50.0 mM until suspended, added 120.0 µL lisozyme 10 mg/mL, and incubated at 37ºC for 45 minutes. The mixture was centrifuged at 12,000 rpm for 5 minutes. Pellet was added 600.0 µL of Nucleic Lysis Solution, incubated at 80°C for 5 minutes, cooled to room temperature and added 3.0µL RNAse. The sample was homogenized for 2-5 minutes and incubated at 37°C for 45 minutes. The sample was cooled to room temperature, added 200.0µL of Protein Precipitation Solution, vortexed at high speed for 20 seconds and incubated in ice for 5 minutes. The sample was centrifuged at 12.000 rpm for 5 minutes. The supernatant was put in the microtube that filled with 600 µL of isopropanol solution. The sample was centrifuged at 12.000 rpm for 5 minutes. The pellet was added by 600 µL of cold ethanol 70% and the microtube was turned over. The mixture was centrifuged at 12.000 rpm for 3 minutes. The supernatant was removed and the pellet was dried using a freeze dryer. Dried pellet (DNA) was added 100 µL DNA Rehydration Solution. The DNA solution was stored at -20°C.

1.3 Electrophoresis Genomic DNA of UKG Isolate

Genomic DNA was electrophoresed using 0.8% agarose gel (0.2 g of agarose was dissolved in 25.0 mL TAE 50 mM). The mixture was boiled in the microwave. The mixture was cooled to temperature of 45-50ºC and added 1 µL of redsafe, then stirred until homogeneous. The agarose solution was put in mold gel. TAE 1X buffer was added until the gel was submerged. Genomic DNA 10 µL was mixed with 2.0 µL of 6x loading dye solution. As a marker, 1 µL ladder DNA 1 kb was mixed with 1 µL of 6x dye loading and 4 µL of 1x TAE buffer. Electrophoresis was carried out for 45 minutes, 75 volts and 400 mA. Gel was observed on UV-transiluminator.

1.4 Isolation of 16S rRNA Gene from UKG Isolate

Isolation of 16S rRNA gene from UKG isolate using PCR method. Total volume of the reaction mixture for amplification process was 50.0 µL which consisted of 39.5 µL of ddH_2O, 1 µL of dNTP mix 10 mM, 1 µL of UniB1 primer 20 µM, 1 µL of BacF1 primer 20 µM, 2 µL samples, 5 µL of Dream Taq buffer 10x (added Mg^{2+}) and 0.5 µL Dream Taq polymerase. The mixture was homogenized for 15 seconds. The initial denaturation process was carried out at 94ºC for 2 minutes. The second denaturation was carried out at 94ºC for 1 minute. Annealing process at 48ºC for 1 minute and elongation process at 72ºC for 1 minute. Post elongation at 72ºC for 10 minutes. The PCR cycle was repeated 30 times

1.5 Cloning of 16S rRNA Gene to pGEM-T *Easy* Vector

1.5.1 Ligase Reaction

Ligation reaction was carried out according to procedure in Promega. Ligation reaction component were 5 µL ligase buffer 2x, 2 µL ddH_2O, 1 µL pGEM-T Easy vector 50 ng/µL, 1 µL ligase enzyme and 1 µL 16S rRNA gene. The mixture was incubated at 4ºC overnight.

1.5.2 Prepared of Competent Cell

Preparation of competent cells was carried according to Sambrook method [13], which modified slightly. The competent cell was made from *E.coli* TOP10F which had been cultured in LB solid medium (Luria Bertani). LB liquid medium (5 mL) was added 5 µL tetracycline 5 mg/mL. *E.coli* TOP10F bacteria were taken using a sterile toothpick and put into LB liquid medium aseptically. LB liquid media was stirred using a shaker at 37°C for 18 hours, 150 rpm. The 200 µL bacterial culture was put into 20 mL LB liquid medium which had been added 20 µL tetracycline 5 mg/mL, then it was taken at 37°C for 3 hours, 150 rpm (OD=0.2-04). Bacterial culture was transferred to cold bottle 50 mL in ice, then incubated for 15 minutes. Samples were centrifuged 2700 g at 4ºC for 10 minutes. The pellet was taken and washed with 5 mL of cold $CaCl_2$ 0.1M and incubated for 10 minutes in ice. The mixture was centrifuged at 2700 g for 10 minutes, 4ºC. Pellet added 0.8 mL of cold $CaCl_2$ 0.1 M. The mixture was incubated in ice for 2 hours.

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1.5.3 Transformation

The transformation procedure was carried according to Sambrook [13] with slightly modified. The 100 µL of competent cell was included in a microtube 1.5 mL that containing 2 µL recombinant pGEM-T Easy vector (as a negative control was without using a pGEM-T Easy recombinant plasmid). The mixture was incubated in ice for 30 minutes, then in water bath of 42^{o}C for 90 seconds for heat shock. The tube was incubated in ice for 2 minutes. The 900 µL LB liquid medium was added and shaked at 37°C for 1 hour, 150 rpm. The 100 µL culture containing recombinant plasmid was taken and spread to LB solid medium which had been added ampicillin, X-Gal and IPTG (negative control was LB solid medium without X-Gal and IPTG). The residual culture of 900 µL was centrifuged for 1 minute, 12,000 rpm. The 800 µL supernatant was discarded and only 100 µL is left. The 100 µL pellet and supernatant resuspended, then dispread on solid medium that had been added to ampicillin, X-Gal and IPTG. Samples were incubated overnight at 37^{o}C. White colonies were transferred to solid medium which had been given ampicillin and incubated at 37°C for 18 hours.

1.6 Isolation of Recombinant DNA

Isolation of recombinant DNA was carried out according to the High-Speed Plasmid Mini Kit method (ATP Biotech). Recombinant DNA was isolated from white colony. Bacterial culture was transferred to a microtube 1,5 mL and centrifuged at 12,000 rpm for 2 minutes. The 200 µL PD1 buffer (added RNAse) was added and resuspended. The 200 µL PD2 buffer was added in microtube. The mixture was allowed to stand for 2 minutes at room temperature. The 300 µL PD3 buffer was added in microtube, centrifuged at 12,000 rpm for 5 minutes, 4^{o}C. The supernatant was taken and transferred to a PD column in a collection tube, then centrifuged 12,000 rpm for 1 minute. The liquid was discarded of in the collection tube and placed in a PD column in the collection tube. The 400 µL buffer was added to the PD column and centrifuged at 12,000 rpm for 1 minute. The liquid was discharged in the collection tube and the PD column was placed in the collection tube. The 600 µL wash buffer (added ethanol) was added into a PD column and centrifuged at 12,000 rpm for 1 minutes. The liquid was discarded of in the collection tube, then centrifuged for 4 minutes to dry the matrix column. The PD column was transferred to a microtube 1,5 mL and 2x25 mL elution buffer was added on the middle of the matrick column. The PD column was allowed to stand 30 minutes until the liquid was absorbed, then centrifuged at 12,000 rpm for 2 minutes. The recombinant DNA was checked by using DNA electrophoresis.

1.7 Restriction Analysis of Recombinant DNA

Restriction analysis of recombinan DNA was done by mixing several reaction components in a microtube. They were 1 µL EcoRI buffer 10x, 1 µL recombinant DNA, 0.1 µL EcoRI enzyme 10 u/µL and 7.9 µL ddH_2O. The mixture was incubated at 37°C for 4 hours. The restriction product were checked by using DNA electrophoresis.

1.8 Sequencing 16S rRNA Gene Using Dideoxy-Sanger Method

Gene of 16S rRNA in recombinant DNA was sequenced using the Dideoxy-Sanger method at Macrogen in Korea.

1.9 Analyze Nucletide Base Sequences of 16S rRNA Gene

Electropherogram data of 16S rRNA gene sequences were read and analyzed using the DNAStar program.

3. RESULTS AND DISCUSSION

3.1 Cloning 16S rRNA Gene to pGEM-T *Easy* Vector

The cloning of 16S rRNA gene aims to get the whole 16S rRNA gene sequence on PCR products. In this study, the cloning process were done ligation reaction, transformation and isolation of recombinant DNA from transformant. The ligation reaction is combination of target DNA molecules with vector. The target gene was 16S rRNA gene which isolated based on the PCR method and the vector was the pGEM-T Easy. The PCR method has also been used to isolate gene fragments that encodes inulin hydrolysis enzyme [11][14]. The pGEM-T Easy vector is a vector that has been cut with the endonuclease enzyme (EcoRV) and followed by the addition of a T residue at both ends of 3'. The target gene was used a PCR product that uses the Taq Polymerase enzyme, so that it has residue A at both ends of 5'. The sequences of pGEM-T Easy vector has ampicillin resistant and the Lac Z region which can be used to verify the success of tranformation [15]. Sequences of T7 and SP6 promotor in pGEM-T Easy vector is shown in Fig.1.

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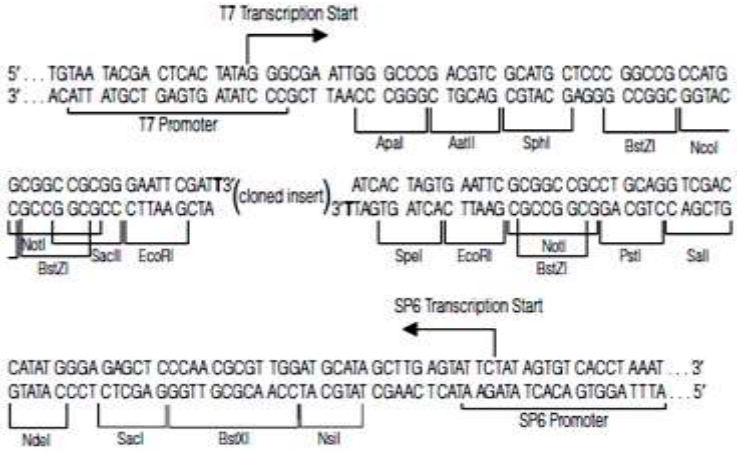

Fig.1 Sequences of T7 and SP6 promotor on pGEM-T Easy vector [15]

Recombinant DNA was transformed to *E.coli* TOP10F cells. Negative charged of DNA plasmids will be attracted to positive charge in surface of competent cell membrane that caused cell treatment using $CaCl_2$. Heat shock causes pores of the outer membrane of the competent cell was stretched, therefore recombinant DNA can enters competent cell. *E.coli* TOP10F containing recombinant DNA was grown on solid medium containing ampicillin, IPTG (isopropyl thiogalactosidase) and X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galacto pyraniside). The success of cloning can be seen through white colonies [13].

Cloning of the 16S rRNA gene has been carried out successfully. There were blue and white colonies on solid medium. Blue-white colonies can distinguish transformant that contained insert DNA (16S rRNA gene) and without insert DNA in recombinant DNA. The existence of blue-white colonies is the application of the Lac operon method carried out by Jacob and Jacques Monod who have the same principle in gene regulation. Blue colonies did not contain insert DNA in pGEM-T, therefore the LacZ can produce ß-galactosidase. IPTG is a lacZ gene inducer. The β-galactosidase catalyzes the breaking of bonds in X-gal to produce galactose and 5-bromo-4-chloro-3-hydroxyindole. This compound will be oxidized to 5.5-dibromo-4.4-dichloro-indigo that is blue compound [16]. White colonies was caused the 16S rRNA gene (insert DNA) separated the LacZ region in white colonies. So, the LacZ can not express ß-galactosidase. Transformant of blue and white colonies were shown in Fig. 2.

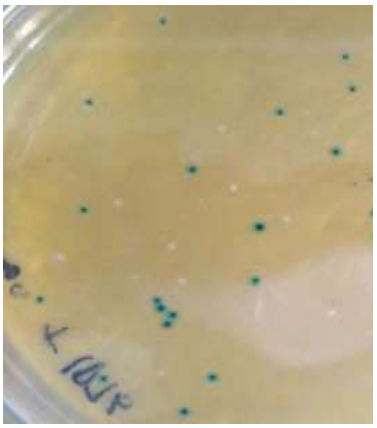

Fig.2 Transformant of White and Blue Colonies

Recombinant DNA was isolated from white colonies using method of ATP^{TM} Plasmid Mini Kit (www.ATP.biotech.com). The isolation process of recombinant DNA involves several stages, harvesting of bacterial culture, lysis of bacterial cell using chemical reagents such as EDTA and detergent, netralization, DNA binding in membran silica column, washing and elution of recombinant DNA. Recombinant DNA were electrophoresed using agarose gel (Fig. 3).Concentrations of recombinant DNA from colonies 1, 2 and 3 were 500 ng/µL, 400 ng/µL and 500 ng/µL. The size of pGEM-T Easy vector was 3000 bp, while the size of 16S rRNA gene was 1500 bp. Therefore, size of recombinant DNA was 4500 bp. Recombinant DNA is circular. Migration of circular DNA is different from linear DNA on agarose gel. Circular plasmids DNA will appear faster than linear plasmids DNA at the same size. Circular plasmids DNA have 2 forms in agarose gel: supercoiled circles and nicked circles [17,18]. In Fig.3 the DNA plasmid is supercoiled circles on agarose gel. Data of plasmid DNA electrophoresis cannot be used to determine the size of plasmid DNA, because the DNA plasmid is circular. So, the migration pattern of circular DNA is different from the linear DNA (DNA marker) on agarose. Therefore, restriction analysis is needed to prove recombinant DNA contains insert DNA (the 16S rRNA gene). Researchers chose the *Eco*R1 enzyme to cut recombinant DNA. The *Eco*R1 recognize the 5'GAATTC3' sequence. *Eco*R1 cut the left and right sides of the insert DNA. Therefore, presence of insert genes can be known. The reaction components

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that used for the restriction analysis were 6 µL *Eco*R1 buffer, 0.1 µL *Eco*R1, 1 µL recombinant DNA and ddH_2O up to volume of 10 µL. The recombinant DNA was hydrolyzed using *Eco*RI produce 1500 bp and 3000 bp in electrophoresis agarose gel. The 1500 bp band was the insert DNA (16S rRNA gene) and the 3000 bp band was the pGEM-T Easy vector.

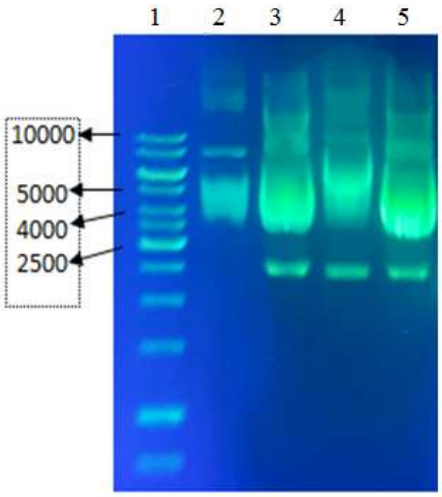

Fig.3 Electrophoresis of Recombinant DNA

(1) DNA Marker, (2) pG, (3) Coloni 1, (4) Coloni 2, (5) Coloni 3

Restriction analyze of recombinant DNA was shown in Fig.4. Recombinant DNA from colony 1 and colony 3 had 5 clear bands (4500 bp, 3000 bp, 1500 bp, 700 bp and 800 bp). Colony 2 had DNA fragments 3000 bp, 700 bp and 800 bp. DNA band 4500 bp is recombinant DNA band, while DNA band 3000 bp is pGEM-T Easy. DNA band 1500 bp in colony 1 and 3 are 16S rRNA gene band. Otherwise, colony 2 did not have 1500 bp band. There is a band measuring 800 bp and 700 bp in agarose gel. The addition of both bands is 1500 bp. It can be assumed that the band is the 16S rRNA gene band. Therefore, the researchers concluded that the 16S rRNA gene has a sequence that is recognized by the *Eco*R1. The band 4500 bp from colony 1 and colony 3 can be concluded that the restriction reaction cut recombinant DNA imperfect. In colony 1 and 3, enzyme that used is not enough to cut all recombinant DNA plasmids. However, the restriction reaction in colony 2 takes place perfectly which had 3 bands.

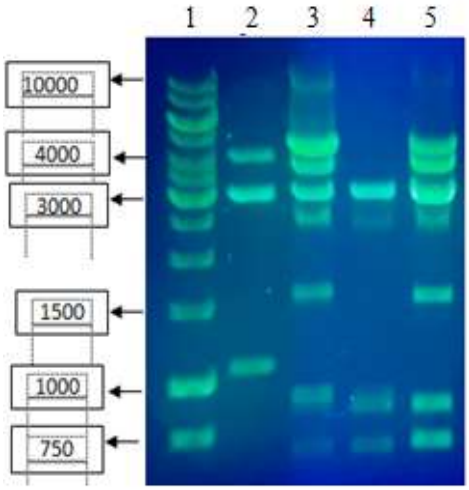

Fig.4 Restriction Analyze of Recombinant DNA

(1)Marker, (2) pG,(3)Coloni 1, (4) Coloni 2, (5) Coloni 3

3.2 Sequencing of 16S rRNA Gene on Recombinant DNA and sequences Analyze

The 16S rRNA gene was sequenced using 2 primers, T7 (5'TAATACGACTCACTATAGGG3') and SP6 (5'ATTTAGGTG ACACTATAG3') primers. Position of PCR primers and sequencing primers on recombinant DNA were shown in Fig.5. Sequencing reactions were started in 61 base sequence for T7 primer and 82 base sequence for SP6 primer. The use of T7 and SP6 primers as the initial sequencing reaction was to obtain the whole 16S rRNA gene sequence. Data of sequence can read well in position about 50-60 bp.

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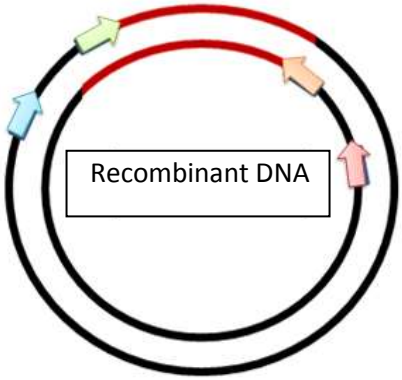

Fig. 5 Map of Recombinant DNA and Primers Position

16SrRNA Gene
UniB1 Primer
T7 Primer
BacF1 Primer
SP6 Primer

Sequencing data were read using the DNAStar program. Electrophoregram fragment can be seen in Fig 6. Reading of the nuclotide base sequence used the DNAStar program with SP6 primers was 1167 bp (Fig.7), while reading of the nucleotide base sequence was 1218 bp using T7 primer (Fig.8). The 16S rRNA gene sequence that used SP6 primer start from the 75 base sequence, while the 16S rRNA gene that used the T7 primer starts from the 51 sequence. *UniB1* primer is reverse PCR primer, while BacF1 primer is the forward PCR primer.

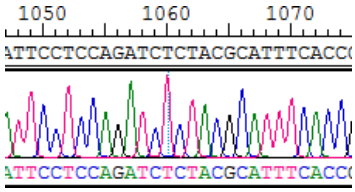

Fig.6 Elektropherogram Fragment Using DNAStar

(-) Base Nucleotide Guanin
(-) Base Nucleotide Timin
(-) Base Nucleotide Adenin
(-) Base Nucleotide Sitosin

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CGCGTTCATG CATCACGCGT TGGGAGCTCT CCCATATGGT CGACCTGCAG   50
GCGGCCGCGA ATTCACTAGT GATTGGTTAC GTTGTTACGA CTTCACCCCA  100
GTCATGAATC ACAAAGTGGT AAGCGCCCTC CCGAAGGTTA AGCTACCTAC  150
TTCTTTTGCA ACCCACTCCC ATGGTGTGAC GGGCGGTGTG TACAAGGCCC  200
GGGAACGTAT TCACCGTAGC ATTCTGATCT ACGATTACTA GCGATTCCGA  250
CTTCATGGAG TCGAGTTGCA GACTCCAATC CGGACTACGA CATACTTTAT  300
GAGGTCCGCT TGCTCTCGCG AGGTCGCTTC TCTTTGTATA TGCCATTGTA  350
GCACGTGTGT AGCCCTGGTC GTAAGGGCCA TGATGACTTG ACGTCATCCC  400
CACCTTCCTC CAGTTTATCA CTGGCAGTCT CCTTTGAGTT CCCGGCCGGA  450
CCGCTGGCAA CAAAGGATAA GGGTTGCGCT CGTTGCGGGA CTTAACCCAA  500
CATTTCACAA CACGAGCTGA CGACAGCCAT GCAGCACCTG TCTCACAGTT  550
CCCGAAGGCA CCAAAGCATC TCTGCTAAGT TCTGTGGATG TCAAGACCAG  600
GTAAGGTTCT TCGCGTTGCA TCGAATTAAA CCACATGCTC CACCGCTTGT  650
GCGGGCCCCC GTCAATTCAT TTGAGTTTTA ACCTTGCGGC CGTACTCCCC  700
AGGCGGTCGA TTTAACGCGT TAGCTCCGGA AGCCACGCCT CAAGGGCACA  750
ACCTCCAAAT CGACATCGTT TACAGCGTGG ACTACCAGGG TATCTAATCC  800
TGTTTGCTCC CCACGCTTTC GCACCTGAGC GTCAGTCTTT GTCCAGGGGG  850
CCGCCTTCGC CACCGGTATT CCTCCAGATC TCTACGCATT TCACCGCTAC  900
ACCTGGAATT CTACCCCCCT CTACAAGACT CTAGCCTGCC AGTTTCGAAT  950
GCAGTTCCCA GGTTGAGCCC GGGGGATTTC ACATCCGACT TGACAGACCG 1000
CCCGGCGTGC GCTTTACGCC CAGTAATTCC GATTAACGCT TGCACCCTCC 1050
GTATTACCGC GGCTGCTGGC ACGGAATTTA GCCGGGGCTT CTTCTGCCGG 1100
GTAACGTCAA TCGAACAGGG TTATTAACCT CACCGGCCTT CCTCCCCGCT 1150
TAAAAGGGCT TTTCAAC                                     1167
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Fig.7 Sequences of Nuclotide Base Using SP6 Primer

UniB1 Primer

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GGTTACGAGT CGCATGCTCC GGCCGCCATG GCGGCCGCGG GAATTCGATT 50
AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG 100
CAAGTCGAGC GGTAGCACAG AGAGCTTGCT CTCGGGTGAC GAGCGGCGGA 150
CGGGTGAGTA ATGTCTGGGA AACTGCCTGA TGGAGGGGGA TAACTACTGG 200
AAACGGTAGC TAATACCGCA TAACGTCGCA AGACCAAAGT GGGGGACCTT 250
CGGGCCTCAT GCCATCAGAT GTGCCCAGAT GGGATTAGCT GGTAGGTGGG 300
GTAACGGCTC ACCTAGGCGA CGATCCCTAG CTGGTCTGAG AGGATGACCA 350
GCCACACTGG AACTGAGACA CGGTCCAGAC TCCTACGGGA GGCAGCAGTG 400
GGGAATATTG CACAATGGGC GCAAGCCTGA TGCAGCCATG CCGCGTGTGT 450
GAAGAAGGCC TTCGGGTTGT AAAGCACTTT CAGCGGGGAG GAAGGCGGTG 500
AGGTTAATAA CCTTGTCGAT TGACGTTACC CGCAGAAGAA GCACCGGCTA 550
ACTCCGTGCC AGCAGCCGCG GTAATACGGA GGGTGCAAGC GTTAATCGGA 600
ATTACTGGGC GTAAAGCGCA CGCAGGCGGT CTGTCAAGTC GGATGTGAAA 650
TCCCCGGGCT CAACCTGGGA ACTGCATTCG AAACTGGCAG GCTAGAGTCT 700
TGTAGAGGGG GGTAGAATTC CAGGTGTAGC GGTGAAATGC GTAGAGATCT 750
GGAGGAATAC CGGTGGCGAA GGCGGCCCCC TGGACAAAGA CTGACGCTCA 800
GGTGCGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACG 850
CTGTAAACGA TGTCGATTTG GAGGTTGTGC CCTTGAGGCG TGGCTTCCGG 900
AGCTAACGCG TTAAATCGAC CGCCTGGGGA GTACGGCCGC AAGGTTAAAA 950
CTCAAATGAA TTGACGGGGG CCCGCACAAG CGGTGGGAGC ATGTGGTTTA 1000
ATTCGATGCA ACGCGAAAGA ACCTTACCTG GGTCTTGACA TCCACCAAAA 1050
CTTAGCAGAA AATGCTTTTG GTGCCTTTCG GGAACTGTGA AAACAGGTGC 1100
TGCATGGGCT GTCGTCCAGC TCCTGGTTGT GAAAATGGTT GGGGTTAAAG 1150
TCCCCCCAAC GAAGCGCAAC CCTTTATCCT TTGTTTGCCA ACCGGTCCCG 1200
GCCCGGGAAA TTCAAAGG                                    1218
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Fig.8 Sequences of Nuclotide Base Using T7 Primer

BacF1 Primer

Sequence of recombinant DNA used T7 and SP6 primers had overlap area. Sequence of 16S rRNA gene in recombinant DNA from the UKG isolate were 1501 bp (Fig. 9). Restriction analysis on 16S rRNA gene sequence had a palindrom sequence that known as *Eco*R1 (GAATTC), which is at least 832-837 nucleotide bases. In addition, the 16S rRNA gene sequence had recognition sequence of *Hind*III (AAGCTT), *Bam*HI (GGATCC), *Bal*I (TGGCCA, *Hae*III (GGCC), and *Sma*I (CCCGGG) restriction enzymes.

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GGTTACGTTG TTACGACTTC ACCCCAGTCA TGAATCACAA AGTGGTAAGC 50
GCCCTCCCGA AGGTTAAGCT ACCTACTTCT TTTGCAACCC ACTCCCATGG 100
TGTGACGGGC GGTGTGTACA AGGCCCGGGA ACGTATTCAC CGTAGCATTC 150
TGATCTACGA TTACTAGCGA TTCCGACTTC ATGGAGTCGA GTTGCAGACT 200
CCAATCCGGA CTACGACATA CTTTATGAGG TCCGCTTGCT CTCGCGAGGT 250
CGCTTCTCTT TGTATATGCC ATTGTAGCAC GTGTGTAGCC CTGGTCGTAA 300
GGGCCATGAT GACTTGACGT CATCCCCACC TTCCTCCAGT TTATCACTGG 350
CAGTCTCCTT TGAGTTCCCG GCCGGACCGC TGGCAACAAA GGATAAGGGT 400
TGCGCTCGTT GCGGGACTTA ACCCAACATT TCACAACACG AGCTGACGAC 450
AGCCATGCAG CACCTGTCTC ACAGTTCCCG AAGGCACCAA AGCATCTCTG 500
CTAAGTTCTG TGGATGTCAA GACCAGGTAA GGTTCTTCGC GTTGCATCGA 550
ATTAAACCAC ATGCTCCACC GCTTGTGCGG GCCCCCGTCA ATTCATTTGA 600
GTTTTAACCT TGCGGCCGTA CTCCCCAGGC GGTCGATTTA ACGCGTTAGC 650
TCCGGAAGCC ACGCCTCAAG GGCACAACCT CCAAATCGAC ATCGTTTACA 700
GCGTGGACTA CCAGGGTATC TAATCCTGTT TGCTCCCCAC GCTTTCGCAC 750
CTGAGCGTCA GTCTTTGTCC AGGGGGCCGC CTTCGCCACC GGTATTCCTC 800
CAGATCTCTA CGCATTTCAC CGCTACACCT GGAATTCTAC CCCCCTCTAC 850
AAGACTCTAG CCTGCCAGTT TCGAATGCAG TTCCCAGGTT GAGCCCGGGG 900
ATTTCACATC CGACTTGACA GACCGCCTGC GTGCGCTTTA CGCCCAGTAA 950
TTCCGATTAA CGCTTGCACC CTCCGTATTA CCGCGGCTGC TGGCACGGAG 1000
TTAGCCGGTG CTTCTTCTGC GGGTAACGTC AATCGACAAG GTTATTAACC 1050
TCACCGCCTT CCTCCCCGCT GAAAGTGCTT TACAACCCGA AGGCCTTCTT 1100
CACACACGCG GCATGGCTGC ATCAGGCTTG CGCCCATTGT GCAATATTCC 1150
CCACTGCTGC CTCCCGTAGG AGTCTGGACC GTGTCTCAGT TCCAGTGTGG 1200
CTGGTCATCC TCTCAGACCA GCTAGGGATC GTCGCCTAGG TGAGCCGTTA 1250
CCCCACCTAC CAGCTAATCC CATCTGGGCA CATCTGATGG CATGAGGCCC 1300
GAAGGTCCCC CACTTTGGTC TTGCGACGTT ATGCGGTATT AGCTACCGTT 1350
TCCAGTAGTT ATCCCCCTCC ATCAGGCAGT TTCCCAGACA TTACTCACCC 1400
GTCCGCCGCT CGTCACCCGA GAGCAAGCTC TCTGTGCTAC CGCTCGACTT 1450
GCATGTGTTA GGCCTGCCGC CAGCGTTCAA TCTGAGCCAT GATCAAACTC 1500
T                                                      1501
```

Fig.9 Sequences of 16S rRNA Gene from UKG Isolate

UniB1 Primer

BacF1 Primer

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4. CONCLUSION

Size of 16S rRNA gene from inulin hydrolyzing bacteria, UKG isolate was 1501 bp. The sequence had palinromic sequence of *Eco*RI restriction enzyme at 832-837. The 16S rRNA gene also has been known by *Hind*III, *Bam*HI, *Bal*I, *Hae*III, and *Sma*I restriction enzymes.

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