

MOLECULAR IDENTIFICATION OF UKG ISOLATE AND CHARACTERIZATION OF ITS INULIN-DEGRADING ENZYME

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ABSTRACT

Inulin-degrading bacteria from the dahlia tuber rhizosphere are potential sources of biocatalyst that hydrolyzed inulin to produce fructose and fructooligosaccharide (FOS). The research was done to identify the UKG isolate that screened from the dahlia tuber rhizosphere and determine the characterization of its extracellular inulin-degrading enzyme. The enzyme was purified using ammonium sulfate. Enzyme activity was determined using DNS reagent. Inulin degrading enzyme from *Klebsiella variicola* UKG isolate worked optimally at pH 5 and 40°C. The enzyme showed endo-type action. The estimated molecular mass of the inulin degrading enzyme was 43 kDa. The enzyme had K_M 63,317 mg/mL, and V_{max} 0,274 $\mu\text{mol/mL}\cdot\text{min}$. The enzyme is suitable for the production of FOS prebiotic from inulin.

Keywords: Inulin, Inulin-degrading Enzyme, 16S rRNA, Endo-inulinase, Exo-inulinase

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INTRODUCTION

FOS and fructose are important compounds for use in the beverage, food, and pharmaceutical industries.^{1,2} Fructose formation from inulin is more efficient than starch. The fructose preparation from starch involves three enzymes: α -amylase and amyloglucosidase catalyzes the hydrolysis of starch to glucose, conversion of glucose to fructose using glucose isomerase. The maximum process yields in starch to fructose conversation was about 42% fructose, 50% glucose, and the remaining 8% oligosaccharides.³ Meanwhile, fructose production from inulin involves one enzyme with fructose yield up to 98%.³ Therefore, inulinase and levanase are very important enzymes for the production of FOS and fructose from inulin.

Generally, inulinase and levanase can be active on inulin, levan, and sucrose substrates. Inulinase and levanase have endo- or exo- action type which could result in different products. Fructose can be produced from inulin by exo-inulinase or exo-levanase, whereas FOS is obtained from inulin by endo-inulinase or endo-levanase. The synergistic effect of endo- and exo- type combination can be used to produce fructose from inulin.⁴

Inulinase from *Bacillus polymyxa* can be active on sucrose, levan, raffinose, and inulin substrates.⁵ Exo-inulinase of *Aspergillus awamori* can hydrolyze the β -(2 \rightarrow 1) or β -(2 \rightarrow 6) bonds on fructooligosaccharides (inulin and levan with DP 4-7).⁶ Levanase that expressed in *Escherichia coli* of *Bacillus subtilis* was active on levan, inulin, and sucrose substrates.⁷ These substrates can be hydrolyzed using exo-levanase from *Gluconacetobacter diazotrophicus* SRT4.⁸

Fungi, yeast, and bacteria are potent sources of inulinase but bacteria are a scarce source compared to yeast and fungi.⁹ Isolation and characterization of bacteria that had inulinase activity from soil samples were first reported by Allais et al., in 1986. Most of the bacteria that had been found were *Flavobacterium multivorum*.¹⁰ Inulin-degrading bacteria have been isolated from some extreme places such as hot springs, high pH environment, and deep seabed. *Actinomycece Nicardiopsis* sp.DN-K15

was isolated from marine sediments Jiaozhou Bay China expressed alkalitolerant inulinase with optimum activity at 60°C, pH 8, and active in the broad pH range of 5 to 11.¹¹ Furthermore, inulinase of *Sphingobacterium* sp GN24 had activity at low temperature.¹²

Inulin-degrading bacteria that were identified as *Bacillus licheniformis* were screened from Bukik Kili hot spring in Solok (West Sumatra). The gene fragment encoding levanase from the *Bacillus licheniformis* has been isolated by PCR method using DPE.sIF and DPE.eR primers.¹³ The gene fragment from *Bacillus subtilis* was isolated using the same PCR primers.¹⁴ The protein fragment of the gene had conserved motif FSGS. *Paenbacillus* sp LX16 isolated from the root of *Jerusalem artichoke* can degrade inulin to produce FOS.¹⁵ Inulin-degrading bacteria from the dahlia tuber rhizosphere have been screened and identified. In this paper, molecular identification of bacteria UKG isolate and biochemical characteristics of partially purified extracellular inulin-degrading enzymes were described.

EXPERIMENTAL

Raw Inulin

Inulin as a substrate of the inulin-degrading enzyme was extracted from dahlia tubers using ethanol and hot water. The method from Wack et al., 2006 is slightly modified.¹⁶ Inulin was characterized using FTIR. Inulin from *Chicory* was as standard (Sigma, Aldrich).

Screening of Inulin Degrading Bacteria

UKG bacteria isolate was screened from rhizosphere dahlia tuber that is grown in Solok, West Sumatra using a media that contained inulin as the only carbon source. Composition of media was (g/L); 14g KH₂PO₄, 6g K₂HPO₄.3H₂O, 2g (NH₄)₂SO₄, 1g trisodium citrate, 0,2g MgSO₄.7H₂O, and inulin.¹⁷

Identification of Bacteria

The identification of inulin-degrading bacteria was carried out based on 16S rRNA gene sequences. Bacterial genomic DNA was isolated and purified according to the procedure of the Wizard Genomic DNA Purification Kit (Promega). The 16S rRNA gene was amplified using the PCR method with BactF1 and UniB1 primers. The master mix composition was as follows: 5 µL 10x Dream Taq buffer, 1 µL dNTP mix 10 mM, 1 µL primer BactF1 (5'AGAGTTTGATC(A/C) TGGCTCAG3') 20 µM, 1 µL primer UniB1 (5'GGTTAC(G/C)TTGTTACGACTT3') 20 µM, sample 2 µL (30 ng/µL), 0.5 µL Dream Taq polymerase (5U/µL) and ddH₂O was added to 50 µL. PCR steps were carried out at 94°C for initial denaturation for 2 minutes, denaturation 94°C for 1 minute, annealing 48°C for 1 minute, elongation 72°C for 1 minute, and final elongation 72°C for 10 minutes. PCR cycle was performed 30 times. The amplicon was electrophoresed on 0.8% agarose gel, 75 volts for 45 minutes. Amplicon was purified according to the procedure in the DNA Fragments Extraction Kit (ATP Biotech Inc).

Fragment of 16S rRNA gene (about 1500 bp) was ligated on vector of the pGEM-T Easy (Promega) and introduced into *E.coli* TOP10F by heat shock. White colonies of *E.coli* TOP10F were grown on LB media for 16-18 hours at 37°C. The composition of media were 80 µg/mL X-gal, 0.5mM IPTG, and 100 µg/mL ampicillin. Insert DNA on recombinant DNA was isolated from the white colony according to the procedure in the High-Speed Plasmid Mini Kit (ATP Biotech Inc). The recombinant DNA was used as templates in nucleotide sequences of the insert DNA using 3 primers namely SP6, T7, and 357F primers. Nucleotide sequencing was carried out by Macrogen (Korea). The phylogenetic tree was created using the Mega Program.

Production of Enzyme

UKG isolate was grown in 200 mL liquid media containing only inulin as a carbon source.¹⁷ Extracellular enzymes were extracted from the culture of UKG bacterial isolate on stationary phase (about 72 hours) by centrifugation at 8,000 rpm (9.820 g, JA14 rotor of Becman), at 4°C for 30 minutes. Extracellular enzymes were concentrated from 950 mL to 250 mL using ultrafiltration.

Partial Purification of Enzyme

Partial purification of the enzyme was carried out according to Elyachioui et al. 1992 with modification¹⁸. Protein was precipitated using 0-80% saturated ammonium sulfate, and left overnight. The mixture was centrifuged at 12,000 rpm (22.100 g, JA14 rotor of Beckman), at 4°C for 30 minutes.

The precipitated protein was dissolved in 50mM acetate buffer pH 5 and dialyzed using a cellophane membrane at 4°C.

Molecular Mass of Enzyme

The molecular mass of the enzyme was determined using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).¹⁹

Inulinase Assay

DNS method with slight modification was used to determinate the amount of reducing sugar²⁰, at λ 500 nm. An aliquot of 50 μ L of inulin substrate (2.5% in acetate buffer) was added by 15 μ L enzyme. The mixture was incubated for 30 minutes, then added by 100 μ L DNS solution. After heating the mixture in boiling water for 15 minutes, it was added to 850 μ L distilled water. One unit of inulinase activity is defined as one μ mol of reducing sugar produced per minute in reaction conditions. Enzyme activity was performed on various pH (3.0, 3.5, 4.0, 4.5, 5.0, 6.0 and 7.0), temperature (20, 30, 37, 40 and 50°C) and substrate concentration (1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50 and 3.00% (w/v)).

Protein Determination

The protein content in partial purification of the enzyme was determined according to the Bradford method.²¹

Determination of Enzyme Action

The determination of enzyme action type was performed on inulin hydrolysis product using Thin Layer Chromatography (TLC) method²². An aliquot of 3 μ L of inulin hydrolysis products was spotted on silica gel 60 F₂₅₄ as the TLC plate. The mobile phase was used as a mixture of butanol, acetic acid, and water (50:25:25, v/v/v). Carbohydrates on TLC plates were detected using 3% urea in butanol, ethanol, water, and H₃PO₄ (0.6 g urea in 16 mL butanol, 1.6 mL ethanol, 1.0 mL water, and 1.4 mL H₃PO₄).

Accession number of 16S rRNA Gene

Nucleotide sequences of the 16S rRNA gene of UKG isolate have accession number MH290480.

RESULTS AND DISCUSSION

Amplification of 16S rRNA Gene, Cloning and Sequencing

The gene of 16S rRNA from UKG isolate has been amplified successfully using the PCR method and purified. The pure amplicon was estimated 1500 bp (Fig.-1). The position of BacF1 primer is the 8-27 base, while the position of UniB1 primer is the 1510-1492 base on the 16S rRNA gene of the *E. coli*.²³ Large concentrations of amplicons may indicate that the 16S rRNA gene is present in many copies in the bacterial genomic DNA. The 16S rRNA gene in the bacterial genome DNA is 1 to 15 copies. About 62% of bacteria have more than one 16S rRNA gene.²⁴

The 16S rRNA gene fragment was cloned on pGEM-T Easy. The plasmid has been linearized using EcoRV in the lacZ area. Approximately 1500 pb pure amplicons were ligated on the vector of pGEM-T Easy. The recombinant DNA was introduced into *E. coli* TOP10F cells by heat shock. *E. coli* transformant was grown in LB media containing IPTG, ampicillin, and X-gal for 14-16 hours. White colonies transformants contained pGEM-T Easy which carried an insert DNA. Blue colonies transformants carried pGEM-T Easy without insert DNA. Recombinant DNA was isolated from the white colony transformant and analyzed using enzyme restriction (data was not shown). Recombinant DNA contains the 16S rRNA gene was analyzed based on restriction enzymes.

Recombinant DNA was sequenced using the Dideoxy-Sanger method with T7, SP6, and 357F primers. The nucleotide base sequence that can be read using the SP6 primer was 1167 bp and there is a UniB1 primer, while the T7 primer was 1218 bp. Both of the sequences overlapped 760 bp. The fragment of the 16S rRNA gene was found 1501 bp in UKG bacteria isolate. The sequence has accession number MH290480 in GenBank. In the sequence of the 16S rRNA gene was found palindromic sequences of EcoRI (GAATTC) at positions 832-837 bases.³⁴

Identification of UKG Isolate Using 16S rRNA Gene

Molecular identification of bacteria was carried out by comparing the sequences of the 16S rRNA gene of the UKG isolate to the 16S rRNA gene sequences from various bacteria loaded on GenBank

using the BLASTn program (<http://www.ncbi.nlm.nih.gov>). The sequences of 16S rRNA gene fragment of UKG isolate have a high similarity with 100 bacterial 16S rRNA genes in the GenBank database. Most of these bacteria are *Klebsiella variicola* with 99% similarity. Thus, it can be concluded that UKG isolates belong to the *Klebsiella variicola* species group.

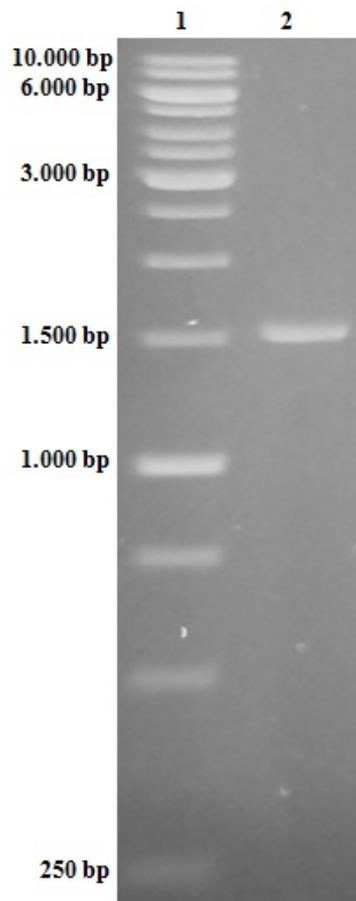


Fig.-1: Electrophoresis of 16S rRNA gene.

Line 1. DNA ladder 1 kb,

Line 2. The 16S rRNA gene of UKG isolate

Analysis of compared nucleotide sequences from 16S rRNA genes can be used to construct phylogenetic trees and can be used as a classification of living things. The use of 16S rRNA gene analysis as an approach to the molecular definition of bacteria can show kinship. The relationship between UKG isolates and other bacteria can be shown by phylogenetic trees (Fig.-2). The phylogenetic tree of the 16S rRNA gene sequences showed that UKG isolates had the furthest kinship relationship with *Klebsiella pneumoniae* strain BK13043. UKG isolates have the closest kinship relationship with *Klebsiella variicola* strain GJ3 with a 99,73% similarity degree. The similarity in the sequence of the 16S rRNA gene less than 97% can be considered as a new species²⁵. Thus, UKG isolate was included in the genus *Klebsiella* and species *Klebsiella variicola*. UKG isolate was named *Klebsiella variicola* UKG. The sequences of the 16S rRNA gene from *Klebsiella variicola*-UKG has been deposited in GenBank with the accession number MH290480.

Estimated Mass of Enzyme Molecules

The inulin degrading enzyme of the *Klebsiella variicola* UKG was partially purified by using ammonium sulfate. The inulin-degrading enzyme was analyzed on SDS-PAGE. The molecular mass of the enzyme was estimated 43 kDa (Fig.-3). The band was also visible (very thin) on the enzyme crude. This molecular mass differs from the molecular mass of the inulin-degrading enzyme from other bacteria. Exo-levanase from *Gluconacetobacter diazotrophicus* SRT4 had a molecular mass of 58.4 kDa⁸. Pure endo-inulinase of *Bacillus smithii* T7 was found 47.5 kDa. The exo-inulinase molecular mass of *Bacillus polymyxa* was 55.52 kDa⁵, whereas *Geobacillus stearothermophilus* KP1289 was 56.744 kDa.²⁶ The mass of inulinase has ranged between 28 to 450 kDa.²⁷

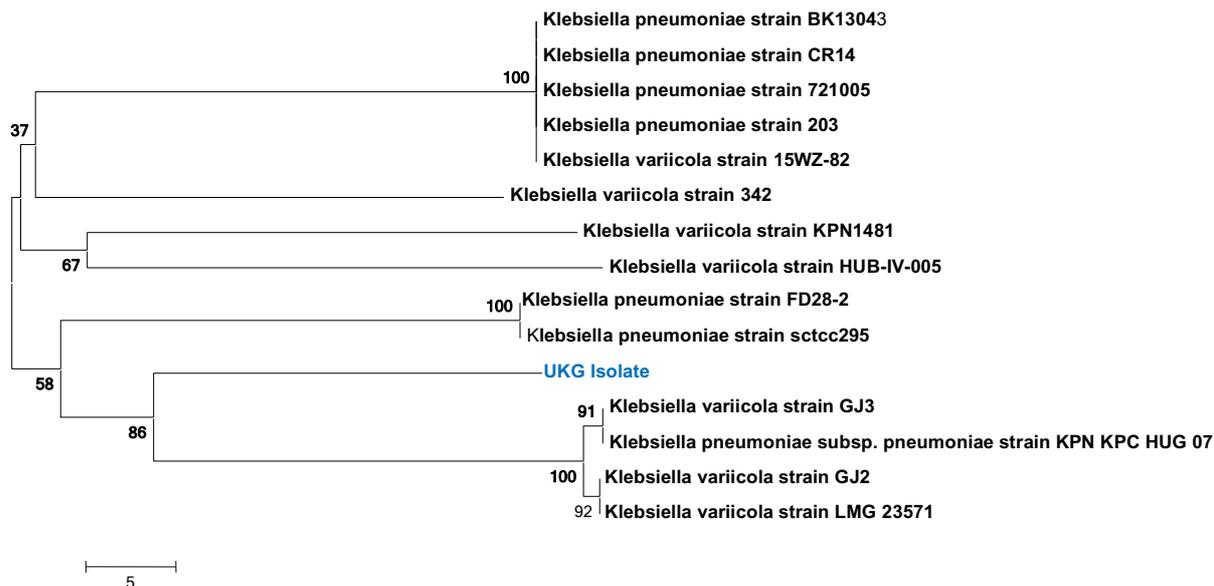


Fig.-2: Phylogenetic Tree of 16S rRNA Gene Sequences from UKG Isolate.

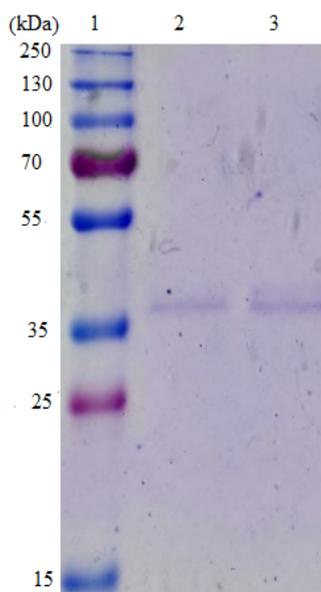


Fig.-3: SDS-PAGE of Inulin-degrading Enzyme from UKG Isolate. Lane 1 was a Protein Marker, Lanes 2 and 3 were partially Pure Enzymes.

The Activity of Extracellular Enzyme on Inulin Substrate

The pH and temperature effect on the extracellular enzyme activity on soluble inulin substrate was carried out in the pH range between 3.0-7.0 and the temperature range between 20-50°C. Partially pure inulin degrading enzyme of *Klebsiella variicola* UKG acted best on inulin substrate at pH 5.0. At less pH 4.5, its activity on the inulin substrate decreased sharply. Thus, the enzyme worked optimally at pH 5.0 (Fig.-4).

The inulin-degrading enzyme of the UKG isolate worked well in the 30-40°C at pH 5.0 (Fig.-5). It rose sharply at a temperature of 20°C to 40°C. The enzyme activity at optimum temperature was 40°C, pH 5.0 at 30 minutes reaction. Thus, maximum reducing sugar was released from inulin in pH 5.0 at 30 minutes reaction.

The optimal temperature and pH of inulin-degrading enzyme from *Klebsiella variicola* UKG is different from inulin degrading enzyme from other bacteria. Levanase from *Bacillus subtilis* shows the most active between temperature 47°C and 55°C with optimum pH between 5.0 and 6.5 on the inulin substrate⁷, while the exo-levanase activity of *Gluconacetobacter diazotrophicu* SRT4 is maximum at pH 6.0 and temperature 30°C.⁸

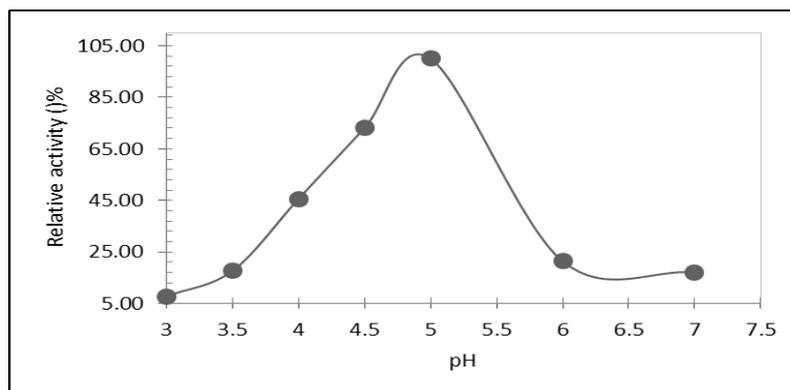


Fig. -4: The Effect of pH on Partial Purified Inulin-degrading Enzyme. The effect of pH was tested using 0.1 M acetate buffer at temperature of 37°C.

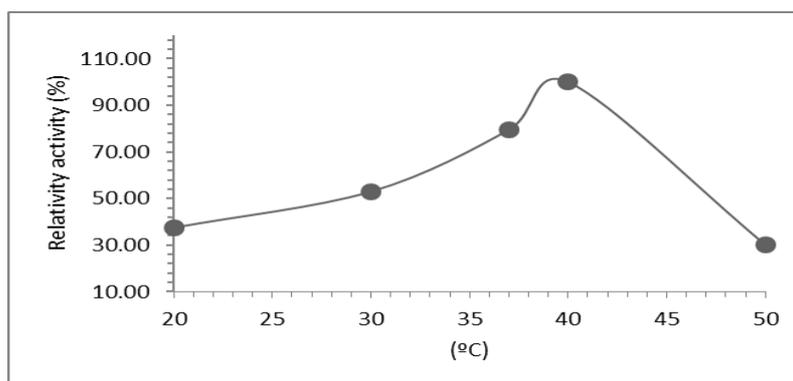


Fig.-5: The Effect of Temperature on Partially Purified Inulin Degrading Enzymes. The Temperature Effect was tested using Buffer of 0.1 M Acetate pH 5.0

The exo-inulinase of *Geobacillus stearothermophilus* KP1289 acts optimally at 60°C.²⁶ Exo-inulinase of *Bacillus* sp.snu-7 has optimal activity at 50°C, pH 7.0.²⁸ Exo-inulinase from *Bacillus polymyxa* has optimal inulinase activity at 35°C and pH 7.0.⁵ In this study, it was obtained that K_M of inulin degrading enzyme from *Klebsiella variicola* strain UKG was 63,317 mg/mL, while its V_{max} was 0,274 $\mu\text{mol/mL}\cdot\text{min}$. Lineweaver-Burk plot of inulin-degrading enzyme from *Klebsiella variicola* strain UKG is shown in Fig.-6. Endo-inulinase of *Xanthomonas campestris* has K_M and V_{max} values on inulin substrate 1.15 mg/mL and 0.15 $\mu\text{M}/\text{min}$, respectively.²⁹

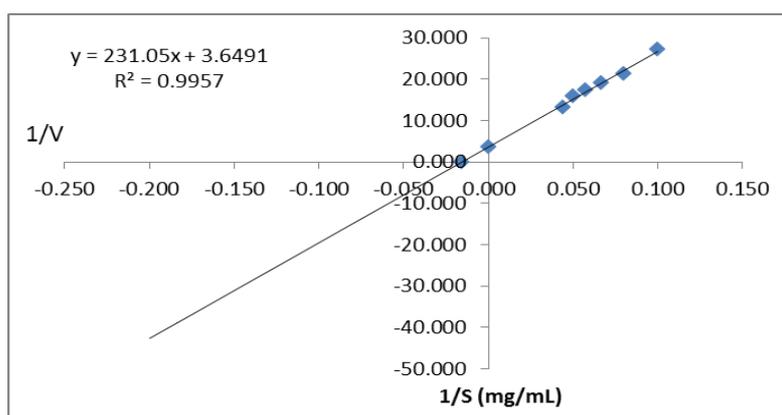


Fig.-6: Lineweaver-Burk Plote of Inulin-Degrading Enzyme

Action Type of The Enzyme

The action type of the enzyme from *Klebsiella variicola* UKG on the inulin substrate was determined by analyzing the inulin hydrolysis product using the TLC method. Zhou et al., and Gill et al., have reported the TLC method for determining the action type of inulinase^{12,22}, while Menendez reported the TLC method for determining the action type of levanase on inulin and levan substrates.⁸

The inulin hydrolysis reaction was carried out for 3.5, 40, and 45 hours at 40°C, and pH 5.0. The analysis of inulin hydrolysis products on the TLC plate showed that the end product of inulin hydrolysis had the same Rf with Rf of standard fructose. Also, the product of in vitro hydrolysis

results showed that there were some spots with Rfs below Rf of fructose. It was interpreted that the hydrolysis products contained FOS with varying sizes. (Fig.-7). FOS is a prebiotic that is added to food to produce functional food. Functional food is important in maintaining our health.³⁰



Fig.-7: TLC Chromatogram of Inulin Hydrolysis Product.
1. Inulin plus pure inactive enzyme, 2. Glucose, 3 Fructose, 4, and 5 were inulin incubated with partial pure enzyme for 3.5, 40 and 45 hours

In the enzyme system of *Klebsiella variicola* UKG was found inulin-degrading enzyme that attacks the internal glycoside bond of inulin to produce FOS. It is known as an endo-type action enzyme. *Klebsiella variicola* UKG may contain exo-type action of inulin-degrading enzyme due to fructose obtained after inulin hydrolysis (Fig.-7). Bacteria that produced endo-inulinase are *Bacillus safensis*.³¹ The most stable endo-inulinase was produced by *Bacillus smithii*.³² *Alkalibacillus filiformis* expressed an extremophilic endo-inulinase.³³ Information of endo-inulinase from bacteria is less than from fungi.

CONCLUSION

The UKG isolate was closest to the species group of *Klebsiella variicola*. The species name of the UKG isolate is *Klebsiella variicola* UKG. The extracellular enzyme characteristics of the *Klebsiella variicola* UKG from the dahlia tuber rhizosphere were performed on the soluble inulin substrate. The inulin-degrading enzyme activity was best at 40°C and pH 5.0. It had an endo-action type and molecular mass of 43 kDa. The enzyme is suitable for the production of FOS prebiotic from inulin.

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