Isolation and identification of exopolysaccharide-producing endophytic fungi from leaf midribs of oil palm

Yurnaliza* and I Jamilah

Department of Biology, Faculty Mathematics and Natural Sciences, Universitas Sumatera Utara, Indonesia, 20155

*E-mail: yurnaliza@usu.ac.id

Abstract. Exopolysaccharide are produced by several types of fungi as well as endophytes. This is the first report about fungal endophytes from oil palm as a exopolysaccharide producer. The aim of this study was to isolated and identified the exopolysaccharide-producing endophytic fungi from leaf midrib of oil palm. Leaf midrib was collected from oil palm in Kuala Bekala Plantation in Medan, Indonesia. Endophytic fungal isolates were characterized morphologically and then identified molecularly by amplification of ITS region of ribosomal RNA. Endophytic fungi of exopolysaccharides-producing were selected by cultivated on mineral medium with sucrose as carbon and NaNO₃ as nitrogen sources. Exopolysaccharides were collected, purified and characterized the FTIR spectrum. The isolation results obtained 14 isolates of fungal endophytes; eleven isolates were characterized as Ascomycetes, and 3 isolates as Basidiomycetes. Based on molecular identification were founded eight of Genera; two Genera in Basidiomycetes, (Schizophyllum and Psathyrella) and 6 Genera in Ascomycetes (Pestalotiopsis, Phyllosticta, Curvularia, Nectria, Diaporthe and Nigrospora). The dominant species of Ascomycetes group were genus Pestalotiopsis. Almost all of fungal endophytes were as exopolysaccharides producer strains. The major functional groups of the exopolysaccharides produced by 11 species of endophytic fungi of oil palm were -OH, -CH, -C = C, and C-O-C groups.

1. Introduction

Exopolysaccharide (EPS) is a saccharide biopolymer that produced by microorganisms such as fungi and bacteria. The fungal exopolysaccharide is one of the high economic value of bio-molecule widely used in various fields of industries, such as medicine, health, and foods. Many types of fungus are known to potentially as a EPS producer [1]. Almost all of fungal types are produce different EPS characters in a different growth conditions [2]. Exploration of the fungal species which produce high grade EPS are need to be done and the endophytic fungus present in oil palm has the potential to be use as a source of isolates.

Oil palm were a famous crops plant in Northern Sumatra of Indonesia. Several research has been reported the variety of fungal endophytes lives inside the plant [3, 4]. Isolation and colonization intensity of fungal endophyte in oil palm around Medan was high, counted 0,99 and 77,1%. In North Sumatera especially in Medan city, the fungal endophyte in oil palm tissues were dominated by species of Ascomycetes. Three genera of fungi in oil palm were potentially to be exopolysaccharide producing, such as genus Lasiodiplodia, Pestalotiopsis, Diaporthe [5]. [2] reviewed the species of filamentous and yeast in phylum Ascomycota and Basidiomycota fungi as exopolysaccharide producer.
in some laboratory condition and medium. Research about application of fungal endophytes from oil palm as a metabolite producer in industrial purpose are rare. Exploration of fungal endophytes in oil palm were conducted to control basal stem rot disease agent, *Ganoderma boninense* [5, 6]. The potency of endophytic fungi as exopolysaccharides producer need to be explored.

The extracellular polysaccharide produced by fungus is a soluble fiber-soluble that has been potential, widely developed and used in various fields of industry. Soluble fiber of polysaccharides are used in food industries, health, medicine, fodder and cosmetics [1]. The genera of fungal endophytes *Diaporthe*, *Marasmius*, *Phlebia*, *Phoma*, *Phyllosticta* and *Schizophyllum* from *Piper hispidum* were reported secreted EPS on the fermentation medium [7]. *Pleurotus pulmonarius* produced EPS in submerged culture using glucose, galactose, arabinose and xylose as carbon sources [8]. *Lasiodiplodia theobromae* reported produce exocellular β-(1→6)-d-glucan (lasiodiplodan) when grown on sucrose [9]. The ability of fungi producing exopolysaccharides is more promising in terms of faster and larger production processes compared to the production of EPS from plants and algae. The commercial fungal polysaccharides product such as Pulullan, Scleroglucan and Botryospaeran. Pullulans produced by *Aureobasidium pullulans* were used as stabilizers in food industry and plastics industry that produces degradable and non-toxic plastics [1]. Exopolisaccharides which was obtained from Ascomycetes and Basidiomycetes fungi were known to possess antioxidant, immunostimulatory, antitumor, and antimicrobial abilities [5]. Many kinds of endophytic fungi were obtained from oil palm tissues and valuable to explore their future benefit. The objective of this study were to obtain and identified the exopolysaccharide-producing endophytic fungi from oil palm tissues.

## 2. Material and Methods

### 2.1 Isolation and characterization of endophytic fungi from oil palm tissues

Leaf midribs of oil palm obtained from oil palm plantation in Medan city of Sumatera Utara Province, Indonesia. The oil palm segments were cleaned with tap water and further surface sterilized with alcohol and sodium hypochlorite [2]. The palm segments were put on sterile medium of Potato Dextrose Agar (PDA ®Merck) medium in room temperature. The emerged of fungal colonies from oil palm segment were collected and put on new agar medium. The growth fungi were characterized of their colony morphology in agar medium.

### 2.2 Identification of Endophytic Fungi

Isolation of Genomic fungal DNA was performed by Promega® DNA isolation kit. The primers ITS1 5' (TCC GTA GGT GAA CCT GCG G) 3' and ITS4 5' (TCC TCC GCT TAT TGA TAT GC) 3' were used for the PCR. Genomic DNA (20 ng) in the PCR reaction was performed as a template. The template were mixed in a 30 µl reaction mixture by using a EF-Taq (SolGent, Korea) as follow: activation of *Taq polymerase* at 95 °C for 2 min, 35 cycles of 95 °C for 1 min, 55°C, and 72 °C for 1 min for each were performed, finishing by a 10-min step at 72 °C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95 °C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). Amplification and sequencing of DNA were performed in Macrogen Inc in South Korea.

### 2.3 Screening of Exopolysaccharide producing fungi

Endophytic fungus grown on EPS production medium was modified from [10]. The carbon source of sorbitol was replaced with sucrose, and pH 6. The medium composition (g.l⁻¹) were KCl (0.5), KH₂PO₄ (1), MgSO₄·7H₂O (0.5) NaNO₃ (3) sucrose (50). Ten plugs fungal mycelium from the margin
of fungal colony (Ø 6 mm) were used as inoculums. The cultures were incubated at room temperature for 10 days in a shaker at 130 rpm. Fungal mycelium was removed by filtration. The exopolysaccharide were collected from medium EPS by ethanol precipitation. Ethanol absolute were used in ratio 1:2 (ethanol:filtrate). Precipitate were collected by centrifugation at 10,000 g for 5 min.

2.4 Analysis of Infrared (IR) spectra

The IR spectrum of exopolysaccharide were analyzed on a Fourier transform infrared spectroscopy (FTIR). Sample of exopolysaccharide were prepared by KBr disk method. A total of 2 mg dried EPS was mixed with 200 mg of dried potassium bromide (KBr). The mixture was solidified in a small container of 16 mm diameter. Spectrum of infra red was read with IR Prestige-21 Shimadzu® instrument (Simadzu-Japan) with wavelength ranging between 400-4000 cm⁻¹.

3. Results and Discussion

3.1 Characteristics of fungal endophytes

Fourteen isolates of fungal endophytes were collected from leaf midribs of oil palm. Identification of fungal isolates was based on colony form or hyphal morphology, characteristics of the discernible spores or reproductive structures [11]. All fungal isolates show the diversity of colony i.e form, color, elevation or margin. The colony dominated by irregular and filamentous form, color white to black and margin undulate, filiform, curled and lobate. Isolate of L4 had similar character to L5 (Figure 1).

For initial growth, some fungal colonies on PDA medium had cottony and becoming darker when aged. Character of mycelial growth on PDA medium were specific to some species, and will be obvious in old culture. Identification of fungal based on morphology of colony was difficult except produced anamorphic conidial. Five colony (L1, L3, L8, L9 and L10) had sporulating conidiomata on PDA medium like a black ink and anamorphic conidial has an appendage-bearing. These conidial character was specific for fungal from family Amphisphaeriaceae, genus Pestalotiopsis [12]. Variations of conidial form can be used to distinguish of fungi from others.

![Figure 1. Morphology colony of fungal endophytes isolates on PDA medium for 7 days](image-url)
3.2 Molecular Identification dan Exopolysaccharide production

Molecular identification of fungal endophytes were used to confirm morphological identification. Analysis of ITS region sequences between small subunit, 5.8S and large subunit rDNA revealed 8 genus of endophytic fungi: Pestalotiopsis, Schizophyllum, Phyllosticta, Psathyrella, Curvularia, Nectria, Diaporthe, and Nigrospora (Table 1). From 14 isolated fungal species, 11 isolates were belonged to Ascomycetes and 3 isolated were basidiomycetous fungi i.e, two isolates of Schizophyllum and one isolate Psathyrella. Two taxa classes in Ascomycetes were Sordariomycetes and Dothideomycetes (Figure 2). Fungal member of both class were habitually lived as an endophytes. Genus Pestalotiopsis was dominant isolated with 3 species; P. theae, P. palmarum and Pestalotiopsis sp. All identified isolates had similarity index ≥99 % to Gene Bank data base of fungi on NCBI site.

Based on exopolysaccharide production, all fungal produced exopolysaccharide except Nectria ipomoeae (isolate L11). The carbon and nitrogen sources for exopolysaccharide-producing medium were sucrose (50g/l) and NaNO3 (3g/l). Pestalotiopsis produced EPS higher than other species. The ability of fungal growth in medium production were not correlated to its exopolysaccharide yield. Nectria ipomoeae isolate was able to growth on medium, but the EPS was not detected in medium. Every fungal will produce variety exopolysaccharide in liquid culture medium according to some parameter. To improve good growth and expolysaccharide production, most researchers had studied optimization of medium production and its physical condition [1, 2, 8].

Table 1. Molecular identification of fungal endophytes isolates from oil palm midrib with primer ITS1-ITS4 and the EPS production.

| No | Code | Gen Bank accession number | Nearest match | Similarity (%) | EPS production | Mycelial dry cell weight (g/100 ml) |
|----|------|---------------------------|---------------|----------------|----------------|-----------------------------------|
| 1  | L1   | JX436804.1                | Pestalotiopsis theae strain C1b5b | 99            | + + +          | 0.6894                            |
| 2  | L2   | KP326577                  | Schizophyllum commune strain UZ1552 14 | 99            | +             | 0.5865                            |
| 3  | L3   | AF409990.1                | Pestalotiopsis palmarum | 99            | +             | 0.661                             |
| 4  | L4   | LN828209.1                | Phyllosticta capitalensis isolate BS5 | 100           | +/-           | 0.3516                            |
| 5  | L5   | LN828209.1                | Phyllosticta capitalensis isolate BS5 | 100           | +/-           | 0.2247                            |
| 6  | L6   | KT273355.1                | Psathyrella candollea voucher EGB12 | 99            | +             | 0.567                             |
| 7  | L7   | HG778982.1                | Curvularia affinis strain CBS 185.49 | 100           | +             | 0.664                             |
| 8  | L8   | -                         | Pestalotiopsis sp | -             | + +           | 0.3096                            |
| 9  | L9   | -                         | Pestalotiopsis sp | -             | + +           | 0.5229                            |
| 10 | L10  | JX436804.1                | Pestalotiopsis theae strain C1b5b | 100           | + + +         | 1.318                             |
| 11 | L11  | AB513849.1                | strain: MAFF 238974 Nectria ipomoeae | 99            | nd            | 0.621                             |
| 12 | L12  | AB369910.1                | Schizophyllum commune strain: IFM 45818 | 99            | +/-           | 0.593                             |
| 13 | L13  | KT964565.1                | Diaporthe phaseolorum isolate MIF01 | 99            | +             | 0.2978                            |
| 14 | L14  | KX256179.1                | Nigrospora sphaerica strain Ns-12 | 99            | +             | 0.4528                            |

Note: mg eps/100 ml medium: +/- < 1; + 1-10; ++ 11-50; +++ >50; nd not detected
Neighbor-joining phylogenetic tree between endophytic fungi of oil palm leaf midribs and related fungi based on internal transcribed spacer sequence showed eight clades representing genera of endophytic fungi. The all clades had 100% of bootstrap which cluster with sequences from genera in NCBI. All fungal endophytes isolates were grouped in three classes of Sordariomycetes, Dothideomycetes and Agaricomycetes. Sordariomycetes and Dothideomycetes were phylum of Ascomycota and Agaricomycetes was Basidiomycota.

3.3 Infrared (IR) spectra

The exopolysaccharides functional group of 11 species of endophytic fungi had -OH, -CH, -C= C, and C-O-C groups (data not shown). Comparison of two FTIR spectrum from two exopolysaccharides from different species fungi shown a similar character (Figure 3). Peak region had a broad stretching in 3375 cm⁻¹ (Figure 3A) and 3383-3382 cm⁻¹ (Figure 3B). Presence of O-H group was detected strong and wide stretching peak between 3600-3200 cm⁻¹ [13]. The hydroxyl groups of carbohydrate is responsible to character and solubility of EPS [15]. The cyclic C-H stretching of methyl and methylene groups was observed absorption band at 2924 and 2927 cm⁻¹ [14]. The peak region at 1631 cm⁻¹ and 1647 cm⁻¹, indicating of the carbonyl bond (C=O) stretching for carbonyl group. Presence of stretching vibration at 1200-900 cm⁻¹ indicated the presence of C-C, C-O-C and CO. [14] suggested the presence of monosaccharide of glucose, galactose and manose in EPS of Pseudozyma at the band region 983–1200 cm⁻¹. Presence of skeletal mode of glicosidic linkage in EPS were detected in absorption band between peaks at 870-890 cm⁻¹.

![Figure 2](image-url)

**Figure 2.** Neighbor-joining phylogenetic tree between endophytic fungi of oil palm leaf midribs and related fungi based on internal transcribed spacer sequence, reconstructed with Mega5 program with bootstrap value repeated 1000 times.
Figure 3 Functional group of FTIR spectrum of exopolysaccharides from A. Pestalotiopsis theae Isolate L1 and B. Curvularia affinis Isolate L7.
4. Conclusion

Fourteen of fungal endophytes isolated from leaf midribs of oil palm, eleven isolates were Ascomycetes and three were Basidiomycetes. Almost all of fungal endophytes produced exopolysaccharide. The major functional groups of exopolysaccharide polymer constituents were -OH, -CH, -C = C, and C-O-C groups.

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References

[1] Mahapatra S, Banerjee D 2012 Carbohydr. Polym 90 683
[2] Osinska-Jaroszuk M, Jarosz-Wilkolazka A, Jaroszuk-Ścisel J, Szalałapa K, Nowak A, Jaszek M, Oźimek E, Majewska M 2015 World J. Microbiol. Biotechnol. 1 1823
[3] Yurnaliza, Aryantha INP, Esyanti RE and Susanto A 2014 Plant Pathology Journal 13 257
[4] Pinruan U, Rungjindamai N, Choeyklin R, Lumyong S, Hyde KD, Jones EBG 2010 Fungal Diversity 41 71
[5] Yurnaliza 2015 The role of oil palm endophytic fungi to control pathogenicity of Ganoderma Boninense Pat. (Bandung: Institut Teknologi Bandung)
[6] Yurnaliza, Aryantha INP, Esyanti RE, Susanto A 2017 Plant Omic Journal 10 247
[7] Orlandelli RC, Vasconcelos AFD, Azevedo JL, da Silva MLC, Pamphile JA 2016 Biochimie Open 2 33
[8] Smiderle FR, Olsen LM, Ruthes AC, Czelusniak PA, Santanafilho AP, Sassaki GL, Gorin PAJ, Iacomini M 2012 Carbohydr. Polym. 87 368
[9] Vasconcelos AFD, Dekker RFH, Barbosa AM, Carbonero ER, Silveira JIM, Glauser B, Pereira MS, da Silva MLC 2013 Carbohydr Polym 92 1908
[10] Selbmann L, Onofri S, Fenice M, Federici F, Petruccioli M. 2002 Res Microbiol 135 585
[11] Barnett HL and B.B. Hunter 1998 Illustrated marga of imperfect fungi 4th ed (USA: Prentice-Hall, Inc.)
[12] Watanabe T 2002 Pictorial atlas of soil and seed fungi - morphologies of cultured fungi and key to species. 2nd ed. (Boca Ratón: CRC Press)
[13] Kaur V, Bera MB, Panesar PS, Chopra HK 2013 International Journal of Biotechnology and Bioengineering Research 4 365
[14] Sajna KV, Sukumaran RK, Gottumukkala LD, Jayamurthy H, Dhar KS, Pandey A 2013 International Journal of Biological Macromolecules 59 84