

Phenotypic and genotypic characteristics of exopolysaccharide-producing fungi as a source of food additives

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Abstract. Akroman R, Nurhayati N, Suwasono S, Jayus J. 2019. Phenotypic and genotypic characteristics of exopolysaccharide-producing fungi as a source of food additives. *Biodiversitas* 20: 2468-2474. As the demand for materials suitable for edible film in food processing is increasing, the exploration of exopolysaccharide (EPS) producing fungi is important. Some fungi had been reported to produce extracellular polysaccharide having rheological properties potential as thickener and or plasticizer useful for edible film raw material. Therefore, this study was focused on isolation and identification of EPS-producing fungi from the plantation area surrounding the University of Jember, Indonesia. The fungal isolate obtained was morphologically identified based on mycelia and spore type, while its genotype was determined using ITS1 and ITS4 primers. The optimum initial culture pH for EPS production was measured using shake flask culture fermentation. The rheological properties of the EPS were determined using Brookfield viscometer, and surface structure of EPS was observed using a scanning electron microscope (SEM). The result showed that the mycelium of the isolate was reddish-brown and septated, its spores were formed as conidiospores. Internal Transcribed Spacer (ITS) sequence of the fungus showed that the isolate is a new species. Therefore, it was designated as *C. lunata* isolate RJ01. Optimum initial pH for EPS production was pH 6.0, achieved after 5 days fermentation yielded 2.6 g L⁻¹ of EPS. The EPS from *C. lunata* isolate RJ01 have no cytotoxic activity on *vero* cell. The rheological behavior of *C. lunata* EPS was viscous, and its surface structure of EPS was compact indicating its potency as thickening agent and plasticizer, a material required for edible film formation in food industries.

Keywords: *Culvularia lunata*, exopolysaccharide, genotypic, phenotypic

INTRODUCTION

Over the last two decades, the demand for film materials for food packaging is increasing (Ramos et al. 2013) to fulfill the industrial need. However, most packaging materials available are considered as a non-renewable and non-biodegradable, and of course non-environmentally friendly, because it will consequently be the main source of solid waste and environmental pollutant (Parreidt et al. 2018). Effort to solve this problem is, among others, through the application of edible film which is abundantly available as the packaging material made from renewable natural polymers (Gontard and Guilbert. 1994). This includes exopolysaccharide from microorganism (Rodriguez et al. 2017), especially fungi. Some fungi had been reported to have the ability to excrete such materials in high amount (Liu et al. 2016).

As one of the EPS sources (Mahapatra and Banerjee 2012), fungi are currently attractive because they are rich in various biologically active substances (Nehad and Shamy 2010). The exopolysaccharides they secreted have several functions as emulsifiers, stabilizers, film making, viscosifying and gelling agents (Mathur and Mathur 2006; Patel et al. 2014). Some fungal EPS exhibit a rheological property showing a pseudoplastic non-Newtonian behavior, and also non-porous compact surface; potential as

plasticizer for making edible film making. Hence, it has potential application as packaging material (Sajna et al. 2013).

Some fungi have been reported to be able to produce EPS, which are potential as food additives such as *Sporobolomyces salmonicolor* AL1 as thickener or gelatinizer (Pavlova et al. 2004) and *Rizobium tropici* LBMP-C01 as emulsifier (Moretto et al. 2015). However, the EPS yield of those fungi were varied without any clear relationship between the yield and the type of fungal strain (Mahapatra and Banerjee 2012). The physical properties of those EPS were also different depending on the structure of the EPS produced. Therefore, exploration of fungal strain producing high EPS yield is essential. The present study aimed to isolate, and to phenotypically and genotypically identify EPS-producing fungi from the plantation area surrounding the University of Jember (UNEJ), East Java, Indonesia, and the potency of the EPS as a source of edible film.

MATERIALS AND METHODS

Media for isolation of fungi

Media for isolation of the fungi were malt extract agar (MEA; Merck). These media were used to capture and

screen the fungi from the plantation area. Czapek dox broth (oxid) was used as the carbon source for EPS production. Mineral salts solution was formulated according to the method of Pitson et al. (1991) and modified by Jayus et al. (2002) using K_2HPO_4 (oxid) and Na_2HPO_4 (oxid) to optimize the isolate's growth in the czapek dox liquid medium.

Isolation of EPS-producing fungi

The EPS-producing fungi were isolated using the method of Schmid et al. (2001). The sterile MEA was prepared in a Petri dish, placed on the plantation area surrounding the UNEJ for 5 minutes at several points of locations. The medium was then incubated at 28°C for three days, followed by the screening of all the captured strain to obtain some single isolates. Isolation of pure isolate was carried out at the Laboratory of Centre for Development of Advanced Science and Technology (CDAST), the University of Jember, Indonesia. The fungal isolates obtained were then stored on a sterile soil at 4°C.

Phenotypic identification of EPS-producing fungi

The characteristics of mycelia, conidiophores, and conidia were observed using the Riddle and Briggs method (1950). The color of the isolate was observed as described in Methuen handbook of color (Kornerup and Wanscher 1967). The slide culture was prepared according to the protocol described by Kumar et al. (2012) and Astriani et al. (2018). The fungal microscopic features were observed under XSZ-107 binoculars microscope at 400x magnification. One loop of mycelia was inoculated into a sterile droplet of MEA on a preparation glass and incubated at 28°C for four days. The isolate morphology was previously identified (Sumangala et al. 2010; Manamgoda et al. 2012; Marin-Felix et al. 2017; Heidari et al. 2018).

DNA isolation, PCR amplification, and sequencing

Genomic DNA of the fungal isolate was extracted using the ZR Fungal/Bacterial DNA Kit™ and subjected to PCR amplification using ITS1 and ITS4 primers (Barus and Steffysia 2013). The PCR reaction mixture was in a total volume of 25 μ L containing dd H₂O (5 μ L), DNA template (1 μ L), ITS1 and ITS4 primers (0.5 μ L each), PCR buffer KOD FX neo (12.5 μ L), 2mM dNTPs (5 μ L), and KOD FX neo (0.5 units). PCR conditions were: 1 cycle of 3 min initial denaturation at 95°C, followed by 35 cycles of 10 sec denaturation at 98°C, 30 sec primer annealing at 52°C, 45 sec extension at 68°C, and a cycle of final extension period at 72°C. The PCR product was then separated on an agarose gel (2% w/v), stained with ethidium bromide, and visualized under UV light using Chemdoc Gel System (Bio-Rad). The amplified PCR product was then sequenced using bi-directional sequencing. The isolate RJ01 was identified by comparing its nucleotide sequences against the Gene Bank database using BLAST (Basic Alignment Search Tools) and a phylogenetic tree was constructed using neighbor-joining at MEGA 5.05 software (Oslan et al. 2012).

Production of exopolysaccharide

Exopolysaccharide production was carried out under different initial culture pH (4,5,6,7,8) of its fermentation. The mycelium of each of the isolates was inoculated into czapek dox broth medium in 1 L Erlenmeyer flask and incubated in a shaking incubator at 28°C for 6 days (Schmid et al. 2001). Samples were observed every 24 h interval for the biomass and the yield of EPS. Biomass was removed by centrifugation, and the EPS in the supernatant was precipitated using ethanol (96%) and then centrifuged at 5000 rpm for 5 minutes (Ramirez 2016). The EPS was then dialyzed for 24 hours against water to remove any remaining low molecular weight compounds, and freeze-dried using a Virtis Advantage Plus freeze-dryer (SP scientific, Warminster, USA). EPS assay were performed using crude β -glucanases obtained from culture media of *Acremonium* sp. IMI 383068 grown on pustulant as substrate, as described by Jayus et al. (2001 and 2004). The EPS (2 mg/mL) were hydrolyzed by the enzyme in sodium acetate buffer (50 mM pH 5.0) containing the EPS as a substrate at 40°C for 30 min. Reducing sugars released were measured using DNS method of Miller (1959).

Viscosity analysis of EPS

Viscosity of the EPS was analyzed using Brookfield DV-II+ Pro with an SC4-18 spindle at room temperature. Its viscosities were measured under a range of concentration between 0.25 to 1.5%.

Scanning electron microscopic analysis of the EPS

Surface microstructure analysis was performed using a Tabletop Scanning Electron Microscope TM303plus (Hitachi High Technologies Corporation, Japan) at the Pharmaceutical Laboratory, Faculty of Pharmacy, University of Jember, Indonesia. The EPS in the form of fine dry powder was observed under a low vacuum environment at the acceleration voltage of 5.0 kV.

Cytotoxicity analysis of EPS

Cytotoxicity of EPS was observed using normal *vero* cells and EPS concentrations of 15.625; 31.25; 62.5; 125; 250; 500; 1000 μ g mL⁻¹. The *vero* cells (5 x 10⁴ cells) were inoculated first into 96 multi-well plates filled with M199 medium (Permanasari et al. 2016) and Hanks'salt solution (Sigma M9163) (100 μ L). Then, the plates were incubated at 37°C in CO₂ (5%) incubator for 24 hours until cells had attached to the well wall. The aliquots of EPS sample of the different concentrations were then added (100 μ L), and the wells were incubated for another 24 hours. To measure cell viability, proliferation, and activation, 3-(4, 5-dimethyl thiazolyl-2)-2 and 5-diphenyltetrazolium bromide (MTT) (100 μ L) were added and further incubated for 2-4 hours at room temperature. SDS (10 %, 100 μ L) in HCl (0,01 N) was then added to stop growth of *vero* cell. Plates were wrapped in aluminum foil and incubated in the dark overnight. An ELISA plate reader, set at 595 nm wavelength, was used to measure the cell viability by comparing the absorbance of sample wells to the control wells.

Statistical analysis

The replicated data were expressed as mean \pm SD (standard deviation). Analysis of cytotoxicity data was carried out using SPSS 17.0, where the P values were determined by one-way ANOVA, followed by the Tukey test.

RESULTS AND DISCUSSION

EPS-producing fungi isolated from the plantation area of UNEJ surrounding

The unscreened fungal colony captured by MEA on the plate appeared colorful as can be seen in Figure 1. Twenty-five single fungal colony were obtained from the screening process of several plates collected from several locations in the agriculture plantation area surrounding the University of Jember, Indonesia. When grown on czapek dox broth for 6 days, one of the single isolates tested was producing an exopolysaccharide, a mucus-like material termed as exopolysaccharide (EPS), secreted into the culture medium, as shown in Figure 2. The hydrolysis of the EPS using β -glucanases, released a non-reducing sugar, glucose, as proof that the mucus-like material is an extracellular polymer of polysaccharide.

Morphological characteristics of EPS-producing fungi

The visual appearance of the colony of the isolate was red, which subsequently turned to reddish-brown when it gets mature. The isolate formed a thin and cottony mycelium (Figure 3). The fungus had an aerial, branched, and septated mycelia, and a regular or irregular border shape (Figure 4.A). Its mycelia form a red colored conidiophore arising singly with simple or branching, septated, bent, and cell walls are thicker than the vegetative hyphae. The conidia are brown, shaped like a boat, circular at the ends, fusiform in shape with three septate, with the brightest end cells, usually bent or curved with enlarged central cells (Figure 4.B). Similar morphological characteristic of this kind of fungi which was identified as *Curvularia* had also been previously reported (Sumangala et al. 2010; Manamgoda et al. 2012; Marin-Felix et al. 2017; Heidari et al.2018).



Figure 1. The appearance of captured fungi after 3 days incubation at room temperature. a. One of the potential EPS producing fungus



Figure 2. Exopolysaccharide appearance after being separated from the culture medium of the isolate

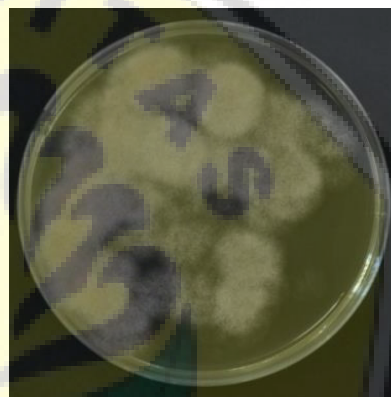


Figure 3. The single EPS-producing fungus isolated from the plantation area of surrounding the University of Jember

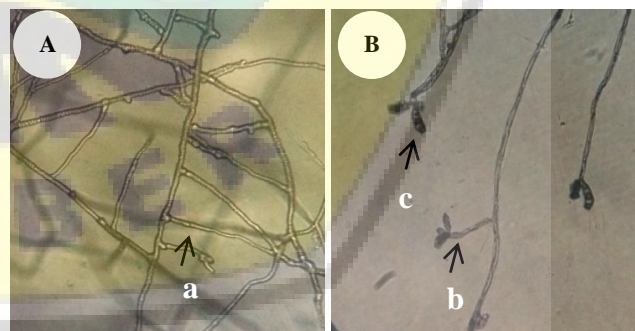


Figure 4. A-B. Morphological characteristic of the isolated EPS-producing fungus under microscopic observation at 400x magnification. Note: a. Septated hyphae, b. Conidiophore, c. Conidiospore

Genotypic characteristics of EPS-producing fungi

The PCR fragments of EPS-producing fungi was approximately 500-750 bp (Figure 5). Results of DNA sequencing of the isolate showed that the ITS region was around 565 bp. The nucleotides data of the isolate analyzed by NCBI blast tool showed the sequence similarity of the isolate of 100% quorum cover with *C. lunata* CY151

(accession number: HQ607991.1) and *C. lunata* strains ITC19 (accession number: KY100122.1). The phylogenetic tree of all isolates shown in Figure 6 divided into three clusters (x; y; z). Cluster x contained *C. aerea* strain 1146, *C. lunata* strain IP 2328.95, *Dothideomycete* sp 7644, *Cochliobolus lunatus* isolate MBC002, and *C. Aeria* strain IP 2328.98 ISSHAM ITS isolate ID MITS1388, *C. Pseudobrachyspora* strain CIMAP: Ac112017, and *C. lunata* clone HW 001. Cluster y contained one species, namely *C. lunata* strain ITC19. Cluster z contained *C. lunata* strain CY151 and the isolated fungus.

The phylogenetic tree analysis based on ITS dataset showed that the closest relative of this isolate is *C. lunata* strain CY151 with a genetic distance of 0.004 and a unique difference of 2 nucleotides in the nucleotide number 519-520. Therefore, this isolate belongs to the species *C. lunata*, which is then designated as *C. lunata* isolate RJ01. Full sequences of the ITS regions of *C. lunata* isolate RJ01 was deposited at the NCBI GeneBank as a new fungus under the accession number of MK629001.1.

Production of exopolysaccharide by *C. lunata* isolate RJ01

C. lunata isolate RJ01 produced an EPS during its growth on czapek dox broth media. The EPS yield increased from the third day until the fifth of incubation time (Figure 7). The highest EPS yield was obtained on the fifth day of fermentation time (2.6 g L⁻¹), and this EPS yield decreased to 1.1 g L⁻¹ on the sixth day of the cultivation time. The decrease of this yield maybe because of the substrate depletion, so the fungus may use the EPS secreted as a carbon source, a similar observation by Yi et al. (2012).

When *C. lunata* isolate RJ01 was grown under different initial pH, the yield was varied depending on the pH of the culture media. Its optimum initial pH was 6.0, with the highest yield of 2.6 g L⁻¹ (Figure 8). This yield might be increased when this fungus grown under controlled pH condition during its fermentation, as has been reported by Sumangala and Patil, 2010) that *Curvularia* sp. was producing higher yield at pH 6. The optimal formation of

EPS by several other fungi such as *Aspergillus ochraceous*; *Bionectria ochroleuca* M21; and *Diaporthe* sp. JF766998 were achieved at pH 6-6.5 (Guo et al. 2014; Li et al. 2016; Orlandi et al. 2016). However, some fungi have been reported to have higher yield at lower pH values such as *Antrodia camphorata* with optimal exopolysaccharide at pH 5 (Shu and Lung, 2004) and *Ganoderma lucidum* at pH 3.5 (Papinutti, 2010).

Viscosity analysis of EPS

When the concentration of EPS in water was elevated from 0.25% to 1.0%, its viscosities were increased two times from 1.01 poise to 2.23 poise, and this also higher compared to that of pullulan (1.79 poise) (Sugimoto, 1978; Tsujisaka et al. 1993) and *Adansonia digitata* mucilage (2.1 poise) in the same concentration of 1% (w/v) (Deshmukh et al. 2013), indicating its high potential for use as a thickener in food industries.

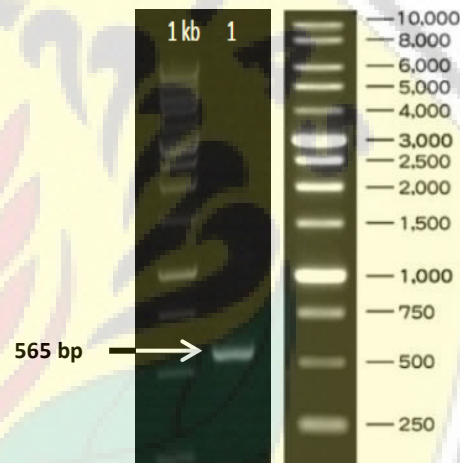


Figure 5. Electrophoresis results of 1 kb DNA Ladder (left), Genomic DNA band of EPS-producing fungi (middle) and 10 kb DNA Ladder (right).

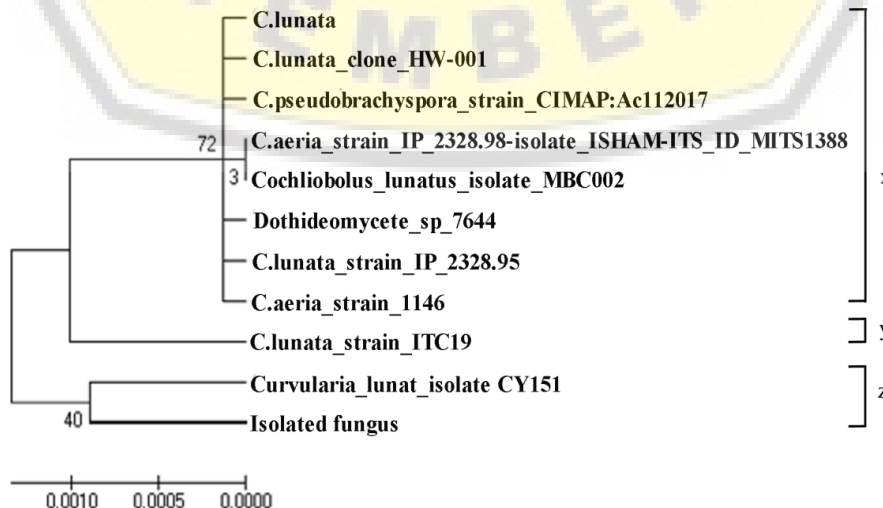


Figure 6. The phylogenetic tree of isolated fungus (*C. lunata* isolate RJ01) using jukes-cantor model

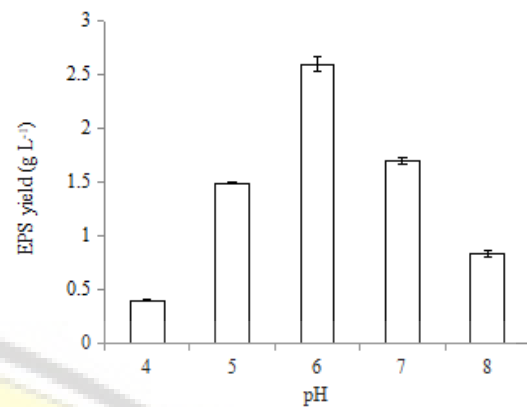
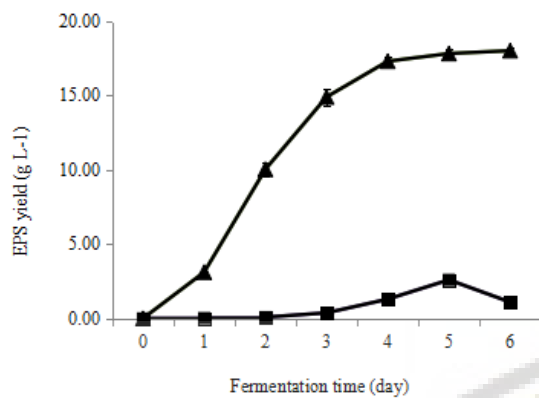


Figure 7. Yield of EPS (■) and biomass (▲) of isolate RJ01 at fermentation days-0 to days-6

Figure 8. The effect of pH on the exopolysaccharide production

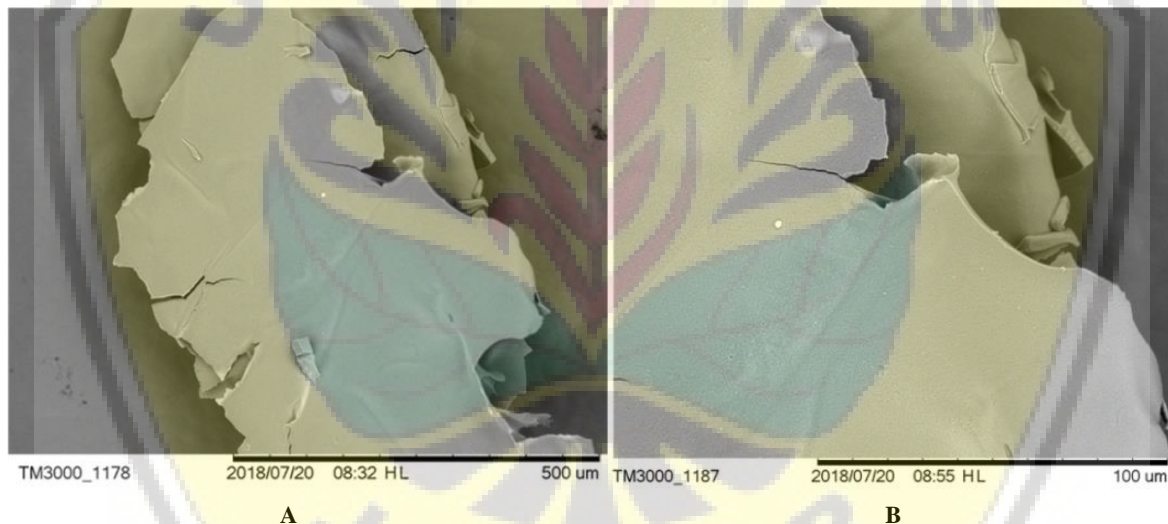


Figure 9. The surface morphology of *C. lunata* RJ01 EPS. Its compact and non-porous film-like structure on 200x (A) and 1000x (B) magnification

Scanning Electron Microscopic Analysis of *C. lunata* isolate RJ01 EPS

SEM analysis on the structure of EPS from *C. lunata* RJ01 showed a typical surface of non-porous film (Figure 9A) and appeared to have a compact structure (Figure 9B). Similar surface structure was reported on the EPS produced by *Pseudozyma* sp. NII 08165 (Sajna et al. 2013). This kind of EPS feature reflects a good potency to be used as plasticized film making materials. Exopolysaccharides are one of the potential polymers for making a bio plasticized film because of its manufacturing process is considered to be environmentally friendly and safe.

Cytotoxicity of EPS of *C. lunata* isolate RJ01

By comparing the growth of *vero* cells in EPS solution with control (0% of EPS), there was no growth inhibition effect, even more *vero* cells were detected in the media containing 15.625 μg mL⁻¹ of EPS (significant difference compared to control) (Figure 10). This indicates that the EPS from *C. lunata* RJ01 did not exhibit any cytotoxicity on *vero* cells. Likewise, in the study of Li et al. (2016) in *Bionectria ochroleuca* EPS, cell viability did not decrease significantly in normal cells treated with EPS with a concentration of 25-1600 μg mL⁻¹.

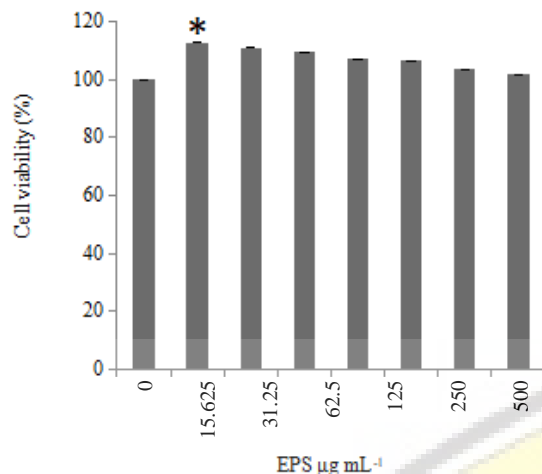


Figure 10. Cytotoxicity effect of exopolysaccharide on *vero* cells. 0 = control (without EPS). Concentration chart marked (*) shows significant difference ($P < 0.05$) of *vero* cells growth compared to control.

In summary, we have isolated and identified *C. lunata* isolate RJ01 and its EPS from the agricultural plantation areas in the University of Jember. The isolate has a reddish-brown color, septate, and conidiospore. Genotypically, this isolate has the highest homology with *C. lunata* strain CY151, and was designated as *C. lunata* isolate RJ01 (accession number MK629001.1). EPS of RJ01 isolate has a non-porous compact surface suggesting its potency to be used in food industry as thickeners and plasticizer, especially on application of edible film making. Future work is needed to investigate the other functional properties of *C. lunata* isolate RJ01 EPS.

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