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***Front cover: Plochoglottis javanica* Blume**
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Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50th annual symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

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Information from internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com

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Structural elucidation of the exopolysaccharide produced by *Curvularia lunata* isolate RJ01

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Abstract. *Jayus J, Akroman R, Nurhayati, Nugraha AS, Piluharto B, Seviour RJ. 2021. Structural elucidation of the exopolysaccharide produced by Curvularia lunata isolate RJ01. Biodiversitas 22: 2699-2705.* An exopolysaccharide (EPS) was recovered from culture filtrates of the fungus *Curvularia lunata* isolate RJ01 prepared in Jember, Indonesia. Based on a prominent peak at 883 cm^{-1}, FTIR analysis suggested it is a *β*-D-glucan, a proposal confirmed by subsequent nuclear magnetic resonance (NMR) analysis which showed anomeric protons omit the signal at δ4.37 and 4.8 ppm, and sugar proton signal in the region of 2.5 to 4.2 ppm. High-performance liquid chromatography (HPLC) analysis of the acid hydrolysates of this glucan revealed the presence of glucose (64%) and a mixture of galactose and mannose (36%). Digestion with (1→3)-*β*- and (1→6)-*β*-glucanase from *Acremonium* sp. IMI 383068 gave consistent linear products of (1→3)-*β*-glycosidic linkages, since the glucan was digested by (1→3)-*β*-glucanase only. The rheological behavior of aqueous EPS solution suggests it behaves as a pseudoplastic non-Newtonian fluid and thus has the potential for use as a thickening agent in foods. This study is the first report on the structural elucidation of EPS from *C. lunata.*

Keywords: *Curvularia lunata*, fungal exopolysaccharide, *β*-glucan, rheological and functional properties

INTRODUCTION

Fungal EPSs are chemically diverse and present a number of possible functions used widely in foods, cosmetics, and medicines (Osińska-Jaroszuk et al. 2020). They exhibit diverse bioactive properties including immunomodulatory, antioxidant, antimicrobial (Mohan et al. 2019), hypolipidemic, hypoglycemic, hepatoprotective and antitumor actions (Wan et al. 2020). An EPS from the fungus *Ganoderma lucidum* has been shown to have multiple biological properties including hepatoprotective, immunomodulating, antitumor, and antioxidant substances (Zhou et al. 2014). Meanwhile, *Sanghuangporus vaninii* polysaccharide has been reported as having antitumor properties only (Wan et al. 2020). Fungal polysaccharides which have immunostimulating effects have been detected in *Cordyceps sinensis* (Wu et al. 2014) and *Grifola frondosa* fruiting bodies (Meng et al. 2017), as well as in other fungi reviewed by Loukotová et al. (2018). Moreover, fungal EPSs also possess antioxidative activities, such as polysaccharides from *Radix pseudostellariae* (Chen et al. 2013), *Auricularia auricula* (Hao 2014), and *Cordyceps sinensis* mycelium and *Ganoderma lucidum* (Zhonghui et al. 2014). However, the relationship between chemical structure and functional properties is still not well understood. For example, *Lachnum* sp. YM26 produces a glucan linked by the β-(1→3)-glycosidic bond and has anti-aging properties (Ye et al. 2012) while the As1-1 from *Aspergillus* sp. Y16 with β-(1→6) and α-(1→2) linkages has strong antioxidant potential (Chen et al. 2011). Furthermore, the rhamnogalactan linked by β-(1→4,6) and α-(1→2) linkages from *Fusarium solani* SD5 has anti-allergic and anti-inflammatory properties (Mahapatra and Banerjee 2012). The *Rhodotorula glutinis* EPS with β-(1→3) and β-(1→4) linkages can act as an anti-viral (Ibrahim et al. 2012), and *Pseudozyma* sp. NII 08165 EPS linked by β-(1→3) has potential for use in thickeners, plasticizers, and emulsifiers (Sajna et al. 2013). Moreover, the exploration and exploitation of fungal polysaccharides show some advantages over other polysaccharides resources, such as distinct and reproducible production parameters to overcome environmental constraints, relatively higher yield, and high quality and purity of the final product (Moscovici 2015).

Fungal exopolysaccharides (EPSs) are carbohydrate polymers that in certain cases are secreted through cell walls and often form a protective layer (Yu et al. 2014; Chen et al. 2013) or, as with many fungal EPSs, are excreted into the environment as mucilaginous slime (Llamas et al. 2012). The polysaccharide monomer units can vary, and include mannose (Chen et al. 2011), glucose

(Guo et al. 2013), galactose (Chen et al. 2010), and rhamnose (Mahapatra and Banerjee 2012), generating either homo- or hetero-polysaccharides that can contain other inorganic or organic compounds (Llamas et al. 2012). Some of these polysaccharides consist of monomers linked by diverse alpha- and beta-glucosidic linkages (Sharmila et al. 2014). Although some are linear, most fungal glucans have a branched (1-3)-ß-linked backbone of glucose units with branches of short glucose chains linked to the backbone by (1-6)-ß linkages in which the frequency of branching can vary (Chen and Seviour 2007; Xiao et al. 2020). Those with beta-linked monomers appear to be less flexible than those whose monomers are linked with alpha glucosidic linkages similar to pullulan (Castellane et al. 2015). However, there is much still to understand with regard to how the structure of EPSs determines their physico-chemical properties. In recent decades research has focused on the relationship between the structure and activity of polysaccharides and their detailed modes of action, with the aim of understanding and using their functional properties.

The use of fungal EPSs in food processing has attracted interest due to their immunostimulating or therapeutic properties, such as hypocholesterolemic and hypoglycemic actions, which may lead to the development of novel functional foods or nutraceuticals (Giavasis 2013). In the food industry, many fungal EPSs have been used for many years, mainly as purified food additives and ingredients for human diet obtained from naturally occurring foods or recovered from metabolites of fungal culture fermentation (Schilling et al. 2020). Due to their diverse and modifiable properties, the spectrum of fungal EPSs applications is wide and includes gelling agents, thickeners, texturizers, suspension stabilizers, and Pickering emulsifiers. Although the physicochemical and biological characteristics and the applications of a number of fungal EPSs, such as pullulan and scleroglucan, have already been well-developed and commercialized, some less industrialized biopolymers, including mushroom polysaccharides such as ganoderan from *Ganoderma* sp., zymosan, lentinan from *Lentinus edodes*, grifolan and epiglucan from *Epicoccum* sp., are still being explored (Toukach and Egorova 2016). As a result, many new fungal polysaccharides continue to appear as the exploration of polysaccharide-producing strains continues. Observation of the roles of polysaccharides in fungi and a deep understanding of the relationship between structure and bioactivity will assist efforts to develop biomaterials both as antitumor drugs and for vaccine production (Barbosa 2020).

Curvularia is an endophytic fungus (Avinash et al. 2015) that is pathogenic to many plants, especially grasses (Manamgoda et al. 2012). In humans, this fungus was found in a person who had persistent fungal endophthalmitis (Alex et al. 2013). Several metabolites of commercial value as antimicrobial agents have been isolated from *C. lunata,* including curvulalic acid and lunatin (Abdel-Ghany et al. 2015), ethyl acetate extract (Avinash et al. 2015) and anthraquinones (Jadulco et al., 2002), curvularin (Mondol et al. 2017), and perylenequinones (Cruz et al. 2020). *C. lunata* strain RJ01 has recently been reported to produce an EPS (Akroman et al. 2019), with potency as a food additive. In this paper, we investigate the chemical and physical properties of the EPS produced by *C. lunata*. To the best of our knowledge, this is the first report on the structural elucidation and physicochemical attributes of an EPS from this fungus.

MATERIALS AND METHODS

Production and purification of exopolysaccharide from *Curvularia lunata* isolate RJ01

Curvularia lunata isolate RJ01 with accession number MK629001.1. was grown in Czapek Dox broth under the same conditions as those described by Ramirez (2016). Biomass was removed by centrifugation and EPS harvested in the supernatant was precipitated using 96% ethanol and this precipitate was harvested by centrifugation at 5000 rpm for 5 minutes (Akroman et al. 2019; 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).

Fourier transform infrared (FTIR) spectra analysis

Infrared spectra of the EPS were generated using an ATR alpha Fourier transform infrared spectrometer (Bruker Optics Inc., USA) in a chemistry laboratory at the Faculty of Pharmacy, University of Jember, Indonesia. The FTIR spectroscopic analysis began when 5µl dry EPS was mixed with KBr powder, then pounded and pressed into 1 mm pellets and placed on a silicone container. The container was then placed on a micro reader unit. Measurement of transmittance was conducted within the wavelength of 4000 to 600 cm^{-1}.

Nuclear magnetic resonance (NMR) spectroscopy analyses

^1H NMR spectra were recorded at 20 ºC using a 500 Mhz JEOL ECS-500 spectrometer carried out at Tropical Disease Diagnostic Centre, Airlangga University Indonesia, in which the chemical shifts were expressed in ppm. The sample was prepared by dissolving freeze-dried EPS (10 mg) in methanol-d_4.

EPS linkage-type after digestion of EPS with a (1→3)- and (1→6)-*β*-glucanase from *Acremonium* sp.

Both Exo (1→3)- and (1→6)-*β*-glucanases were purified from culture media of *Acremonium* sp. IMI 383068 grown on pustulan as substrate, as described by Jayus et al. (2001) and Jayus et al. (2002), and purified with FPLC (AKTA, Amersham Pharmacia Biotech, Sweden) using an anion exchange column (Sigma) as described by Jayus et al. (2004). EPS hydrolysis was performed with each enzyme in sodium acetate buffer (50 mM pH 5.0) containing the EPS (2 mg/mL) as a substrate at 40 ºC for 30 min. The reducing sugars released were then measured using the DNS method (Miller 1959).

High-performance liquid chromatography (HPLC) analysis

The monomer composition of EPS was determined using Knauer HPLC, RI detector type 1260 (Berlin, Germany), Metacharb 87C column using H_20 as eluent, a flow rate of 0.5 mL/min, and temperature of 85 °C, a technique conducted at the Integrated Testing and Research Laboratory, University of Gajah Mada, Indonesia. A 10 mg sample of EPS was hydrolyzed by 2 M H_2SO_4 at 100 °C for 3 h. The hydrolysate was then neutralized with calcium carbonate and filtered using a 0.45 µm Millex filter prior to injection into an HPLC with an injection volume of 20 µL.

Rheological analysis of *Curvularia lunata* EPS

The rheological behavior of the EPS was analyzed using a Brookfield RVDV-II+ Pro meter with 3 spindles, located at the laboratory of Food and Agricultural Products Process Techniques, Faculty of Agricultural Technology, University of Brawijaya, Indonesia. Flow behavior was measured at 30 °C of 1% EPS by comparing shear stress (Pa) and shear rate (1/s).

Statistical analysis

The replicated data are expressed as mean ± SD (standard deviation).

RESULTS AND DISCUSSION

Monosaccharide composition of EPS from *Curvularia lunata* isolate RJ01

The results of the HPLC analysis of EPS hydrolyzed using acid showed that the EPS of *Curvularia lunata* isolate RJ01 is a hetero-polysaccharide type. This is indicated by the presence of several EPS sugar constituents including glucose, galactose, and mannose. The relative sugar ratio in EPS is 6.4: 3.6 (glucose to galactose + mannose, with retention time values of 10.504 and 11.279 minutes, respectively) (Figure 1). This EPS composition is similar to that of *Pseudozyma* EPS and *Rhizobium* sp. PRIM-18, the relative proportions of sugar of which are 2.4 to 5.0 to 2.6 and 6.1 to 1.8 to 1, respectively (glucose to galactose to mannose) (Sajna et al. 2013; Priyanka et al. 2015). Both of these EPSs have high viscosity with pseudoplastic-type non-Newtonian fluid properties. The bioactive properties of similar sugar constituent types have been reported as potentially accelerating wound healing process *in vitro* (Priyanka et al. 2015). Whether or not the EPS from *C. lunata* isolate RJ01 posses bioactivities has not yet been examined further. The more complex sugar constituent polysaccharide from fungus *Sanghuangporus vaninii* (Wan et al. 2020) consists of not only glucose, galactose, and mannose, but also rhamnose, glucuronic acid, galacturonic acid, glucosamine, galactosamine, xylose, arabinose, and fucose also revealed an antitumor activity. The presence of secreted fungal polysaccharides can be in the form of a layer over the surface of the microorganism, and they can be distinguishable from any polysaccharides occurring within the cell. The major functions of EPSs are thought to be as general physical protection avoiding access to any harmful compounds; they may also prevent dehydration. Phagocytoses by other microorganisms may also be prevented by this kind of capsular polysaccharides (Lei and Edmund 2017). Fungal polysaccharides may also play a crucial role in the reproduction of fungi (Barbosa 2020).

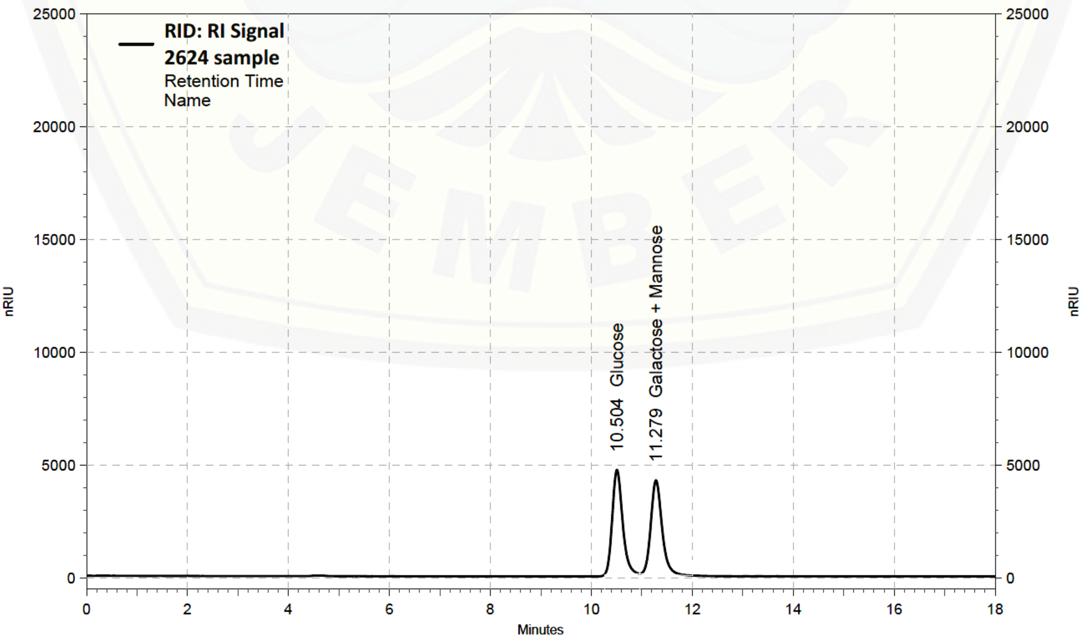

Figure 1. HPLC analysis of *Curvularia lunata* RJ01 EPS

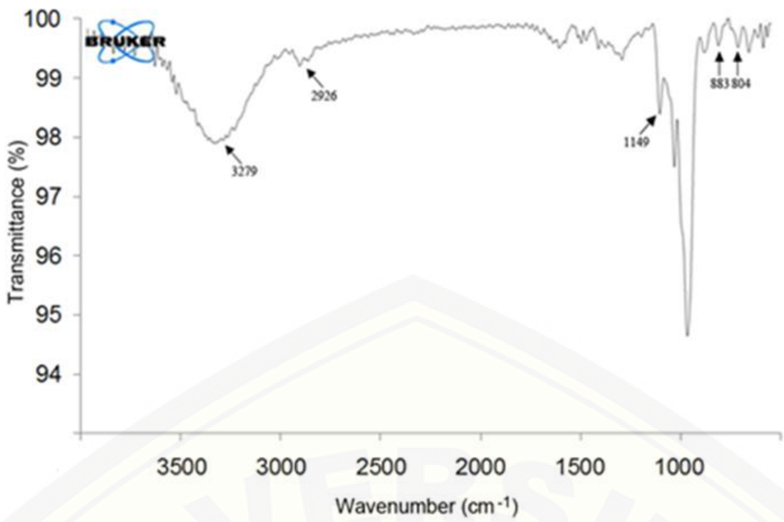

Figure 2. The FTIR spectrum of *Curvularia lunata* RJ01 EPS

Fourier transform infrared spectra of EPS

Our results showed that the functional groups in EPS *C. lunata* were on absorption bands including 3279 cm^{-1}, 1149 cm^{-1} and 2926 cm^{-1} and were in the form of hydroxyl (OH), ketones, or ether (COC) and alkanes (CH) (Figure 2). With the existence of these functional groups, EPS may belong to the group of carbohydrate compounds. Similar results were found in EPS from *Rodotorula* (Ramirez 2016), with its EPS consisting of several functional groups including hydroxyl groups, ketones and alkanes. However, the *Rodotorula* EPS not only contained high total sugar composition made up of glucose and mannose, but also low protein content (Ramirez 2016). The 3279 cm^{-1} hydroxyl absorption band in IR spectra of *C. lunata* showed the presence of polar functional groups of carbohydrate rings which may contribute to the solubility of EPS to water, as reported by Karbowiak et al. 2011. The *C. lunata* EPS spectra showed an absorption band at 883 cm^{-1}, indicating the β configuration of the EPS as observed by Ramirez (2016); Synytsya and Novak (2014); and Yu et al. (2016).

The hydrolysis product of EPS using β-glucanases assay

The (1→3)-β-glucanase assay on the *C. lunata* EPS released reducing sugar as the hydrolysis product, indicating that the EPS consists of (1→3)-β-glycosidic linkages. Meanwhile, in the EPS hydrolysis product digested by (1→6)-β-glucanase, no reducing sugar was detected as the (1→6)-β-glucanase was not able to hydrolyze the EPS. It can thus be assumed that the EPS is a linear β-glucan, different to the other branched fungal β-glucan and fungal cell wall reported by Xiao et al. (2020), and to a rod-shaped polysaccharide such as schizophyllan from the fungi of *Schizophyllum commune* and scleroglucan from *Sclerotium rolfsii*. The *C. lunata* EPS may be similar to a linear random coil type structure such as pullulan from the fungus *Aureobasidium pullulans* (Lei 2016). Given the presence of (1→3)-β-glucan bonds, the 3 glucose monomers, mannose and galactose detected in the HPLC analysis indicate that this EPS structure is linear in shape with no branching. Even though the molecular weight (MW) of the *C. lunata* EPS has not yet been examined, many fungal polysaccharides have been reported as tending to have higher MW (Barbosa 2020). If this is the case with the *C. lunata* EPS, the EPS may have the potential to be used as an adsorber. Linear chain polysaccharides exhibit a greater adsorption ability than branched ones (Lei 2016). A linear neutral polysaccharide with larger MW appears to be adsorbed more easily smaller type. Fungal polysaccharides from *Ganoderma applanatum* and *Abortiporus biennis* have also been reported to be used as water-adsorbing materials (Bancerz et al. 2018).

Nuclear magnetic resonance spectroscopy of EPS

Analysis of the ^{1}H-NMR spectrum of *C. lunata* isolate RJ01 EPS showed the presence of proton signals in the region of 2.5 to 4.2 ppm (Figure 3). The proton signal showed a chemical shift of the sugar proton signals (Nugraha et al. 2015). Chen et al. (2016) and Ge et al. (2013) also observed several EPS proton signals on the spectrum of water-soluble polysaccharides from *Alternaria* sp. and *Phellinus baumii* Pilát that appeared in the region of 3.4 to 4.3 ppm. Additionally, 3 signals of anomer protons appeared at 5.24, 5.18 and 4.20 ppm (labeled as a, b and c, Figure 3). In addition, only an anomeric proton at 4.20 ppm indicated a clear coupling constant value of 6.4 Hz which represents the *β*- configurations, as also observed by Zha et al. (2015) and Zhang et al. (2016). The peak labeled a and b did not provide a well-resolved multiplicity as compound with numbers of hydroxyl moiety (i.e sugars) produced less resolved proton spectra under methanol-*d* as solvent.

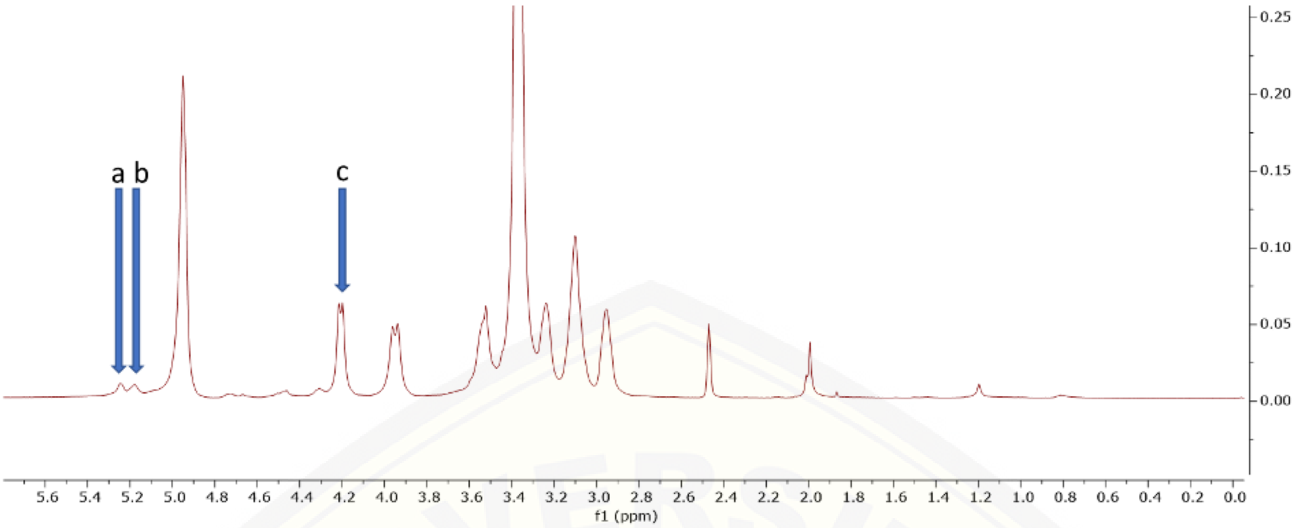

Figure 3. ^{1}H-NMR spectrum of *Curvularia lunata* RJ01 EPS (methanol, 500 MHz)

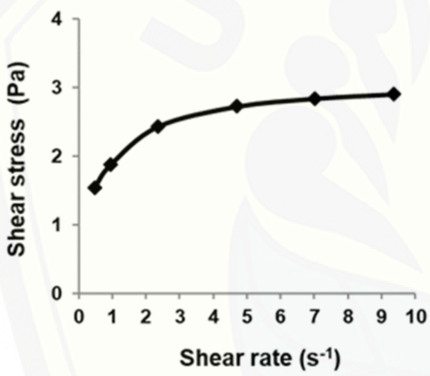

Figure 4. The rheological behavior of *Curvularia lunata* RJ01 EPS

The rheology of *C. lunata* isolate RJ01 EPS

Based on the rheological test of *C. lunata* isolate RJ01 EPS in relation to the shear rate and shear stress at a temperature of 30 ºC, it can be seen that solution viscosity decreased with the increasing rate of shear stress (Figure 4), thus indicating pseudoplastic-type non-Newtonian fluid properties. The EPS was also highly viscous in water (2.23 poise), with a rating higher than that of pullulan (1.79 poise) (Schilling et al. 2020, Tsujisaka and Mitsuhashi 1993) and *Adansonia digitata* mucilage (2.1 poise) in the same concentration of 1% (w/v) (Deshmukh et al. 2013). The high rheological nature of linear *C. lunata* isolate RJ01 EPS in terms of the behavior of non-Newtonian pseudoplastic fluids shows high potential for use as a thickener and gelling agent in various industries. Sajna et al. (2013) also reported that EPS from *Pseudozyma* has potential as a thickener and gelling agent due to its high rheological properties. Some linear β-glucans from bacteria which have been widely used in food and industrial fields, such as curdlan and gellan, are industrially important because of their ability to inhibit syneresis and to stabilize emulsions in dairy products, as well as their ability to control the rheology of water-based systems in other liquid product (Lei and Edmund 2017). The length of the linear chain of polysaccharides will also affect their viscosity in aqueous solutions. As reported by Khan et al. (2017), various molecular weights of β-glucans released by edible mushrooms *Pleurotus ostreatus*, *Agaricus bisporus*, and *Coprinus attrimentarius* exhibit different rheological properties.

In summary, we successfully isolated and identified *C. lunata* isolate RJ01 and its EPS from specimens drawn from agricultural areas in the city of Jember, Indonesia. Based on the identification results derived from FTIR, NMR, HPLC, and enzyme digestion, the EPS is found to have a sugar composition of glucose, galactose, and mannose attached by (1→3)-*β*-glucosidic linkage. This EPS is non-toxic and has a high rheological value and pseudoplastic type, potentially rendering it suitable for use in the food industry as thickeners, emulsifiers, and/or gelling agents. Future work is needed to investigate the bio-functional properties of *C. lunata* isolate RJ01 EPS.

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