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Leclercia adecarboxylata C12, The Newly Isolated Cellulose-degrading Bacteria from Indonesian Coffee Pulp

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ABSTRACT

Culturable cellulose-degrading microorganisms were collected from Arabica coffee pulp in East Java, Indonesia. Fifty isolates were obtained, and thirty-three isolates showed hydrolyzing zone on Carboxy Methyl Cellulose agar plates after Congo-Red staining. The highest specific CMCase activity was observed by isolates C12, identified as *Leclercia adecarboxylata* based on 16S-rRNA gene sequence analysis. SDS-PAGE of *Leclercia adecarboxylata* C12 cellulase revealed two bands with a molecular mass of 95.49 and 81.28 kDa, respectively. Activity gel analysis showed the cellulolytic ability of *Leclercia adecarboxylata* C12 cellulase by clear zone formation. The optimal CMCase activity was achieved at 50°C and pH 9, and the activity retained 47% of its initial activity after incubation at 50°C for 90 minutes. The purified enzyme remains stable from pH 5 to 10, with 77% of its maximum activity. The activity of CMCase was stimulated by the presence of K^+, Ca^{2+}, Mg^{2+}, and Fe^{3+}, while SDS and EDTA reduced its activity. The current study shows that the thermostable-alkalophilic cellulase produced by *Leclercia adecarboxylata* C12 is very promising for industrial applications.

1. Introduction

Microbial cellulase have become the focal biocatalyst due to their complex nature and wide range of industrial applications (Kuhad *et al.* 2011), such as pulp and paper, textile, fruit juice extraction, animal feed additives, and bioethanol production (Bhat 2000). The application of cellulase for industrial applications is increasing until it reaches 20% of global market demand (Jaramillo *et al.* 2015), with the total sales in The United States reaching to 400 million USD per year (Beilen and Li 2002). Likewise, in Indonesia, the use of cellulase has increased by 7% each year (Utami *et al.* 2019). Unfortunately, most of the enzymes for industrial needs (99%) still need to be imported, approximately equivalent to 187.5 billion in 2015 (BPPT 2015). Utilization of potential local resources for enzyme production needs to be done to reduce dependence on imported enzymes in Indonesia. Many studies have been done to find cellulase-producing microorganisms with high specific activity and efficiency when used in industry (Lee *et al.* 2008). Isolation of cellulase-producing microorganisms from high-cellulose agricultural and agro-industrial wastes could help reduce production costs, make them more valuable, and reduce environmental pollution (Wen *et al.* 2005). Coffee pulp is a type of waste that is high in cellulose content. It is often used in biotechnology processes, because it helps create new products (Pandey *et al.* 2000).

Indonesia as one of the world's largest coffee bean producers (BPS 2018) has a high potential for cellulolytic materials. Kalisat Jampit Plantation is one of the largest coffee plantations in East Java with a total production of 840 tons of Arabica coffee beans in 2018. The wet processing process produces coffee pulp up to 42% of the total coffee bean production (Ulloa Rojas *et al.* 2003) or as much as 350 tons of wet weight. It was reported that coffee pulp contains 25.88% of cellulose, 3.6% of hemicelluloses, and 20.07% of lignin (Phuong *et al.* 2019). When Arabica coffee beans are processed, a variety of microorganisms, including bacteria, mold, and yeast, can grow (Feng *et al.* 2016; Junqueira *et al.* 2019; Silva *et al.* 2008). These microorganisms are capable of producing cellulase enzymes, which are

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crucial to the biodegradation of coffee pulp. Each microorganism has specific growth and enzyme production conditions (Elango and Divakaran 2009), so it is necessary to carry out a specific identification process to determine the optimum conditions for enzyme production from the microorganism. This research focuses on the isolation and identification process of cellulolytic microorganisms from Arabica coffee pulp in order to obtain isolates with the highest specific activity of cellulase. The cellulase enzyme obtained was then partially purified through ammonium sulfate precipitation followed by dialysis. Characterization of enzyme under certain condition such as temperature, pH, and the presence of additives was also carried out to determine the optimal conditions for the enzyme activity.

2. Materials and Methods

2.1. Bacterial Growth Condition and Crude Enzyme Production

The cellulose-degrading microorganism C12 was isolated from Arabica coffee pulp of Kalisat Jampit Plantation, East Java-Indonesia Cultures were grown on the CMC agar medium containing CMC 8 g/l, KCl 1 g/l, yeast extract 1 g/l, glucose 1 g/l, $MgSO_4 \bullet 7H_2O$ 0.5 g/l, $NH_4H_2PO_4$ 1 g/l and agar 17 g/l, and incubated on 37°C for 2 days. The grown cultures were then used as starter cultures for CMCase production as described in previous study (Brahmanti *et al.* 2021).

2.2. Identification of Cellulose-degrading Microorganism

The identification of microorganism was carried out using the 16S rRNA method. First, the positive isolates isolated from the chromosomal DNA were extracted using the Quick-DNATM Fungal/ Bacteria Miniprep Kit (Zymo Research, D6005). The concentration and purity of the chromosomal DNA were determined by Nano drop. The amplification of the gene encoding 16S-rRNA was carried out by PCR technique using MyTaq HS Red Mix (Bioline, BIO-25047). The primer used was universal primer 27F and 1492R as describe below:

Forward 27F:5‟–AGAGTTTGATCCTGGCTCAG– 3‟

Reverse 1492R:5‟–TACGGTTACCTTGTTACGACTT– 3‟

The PCR product then purified with Zymoclean™ Gel DNA Recovery Kit (Zymo Research, D4001), and confirmed by 1% agarose DNA gel electrophoresis. Afterward, the PCR products were sequenced so that the sequence of their nucleotide bases would be obtained and the design of the phylogenetic tree of microorganisms with MEGA7 software.

2.3. Effect of Incubation Time on Enzyme Activity and Microbial Growth

Nine ml of CMC broth and 1 ml of culture stock were mixed, then shaken vigorously at 100 rpm for 24 hours at 37°C. The culture was then added to 90 ml of CMC broth, where it was incubated at 37°C and 100 rpm. The CMCase activities as well as the microbial growth were observed every day until 7 days. Measurements of the CMCase activity were carried out by the DNS method (Miller *et al.* 1960), while the microbial growth demonstrated by optical density at 600 nm (Teng *et al.* 2019). Enzyme harvest time was determined by the highest enzyme activity during incubation period.

2.4. Partial Purification of Cellulase Enzyme

The culture supernatant obtained after cellulase production on the optimum incubation period was transferred into an ice cold beaker, as the crude enzyme. The solid ammonium sulphate was slowly added to the crude enzyme until saturation reach 80%, with continuous stirring. The solution then kept in the refrigerator overnight and centrifuged at 10,000 rpm for 20 min at 4°C. The obtained pellet was dissolved in 0.05 mM sodium phosphate buffer (pH 9) (Islam and Roy 2018). The dissolved pellet was then transferred to dialysis bags with a molecular weight cut off of 2,000 (Sigma Aldrich) and dialyzed against 0.025 mM sodium phosphate buffer pH 9 overnight with regular changes of the buffer solution until no more salts were detected by $BaCl_2$ and H_2SO_4 screening.

2.5. Determination of Molecular Weight and Enzyme Activity in Polyacrylamide Gel

Using a Mini PROTEAN II system from BioRad, 10 percent SDS-PAGE gel electrophoresis based on the Laemmli (1970) method was used to determine the molecular weight of the sample. The sample was heated 100°C for 5 minutes in Laemmli loading buffer containing 5% (v/v) 2-mercaptoethanol (2-ME). Electrophoresis was conducted at 80 V for approximately 3 h or until the dye reached the

bottom of the slab gels. The gel was stained with a 0.1% (w/v) Comassie Brilliant Blue R250 solution after electrophoresis, and it was then destaining until the protein bands could be seen. The molecular weight of cellulase was estimated from a plot of the log molecular weight of the standard proteins (iNtRON Biotechnology, Inc.) against elution volume.

Zymogram analyses were carried out by the same SDS PAGE method mentioned above with polyacrylamide gels that copolymerized with 0.1% (w/v) CMC (Sigma-Aldrich) to confirm the enzyme activity. Electrophoresis was conducted at 4°C for 3 h at 80 V in an ice chamber until the dye reached the bottom of the slab gels. The proteins in the gel were renatured with incubation on 2.5% Triton X-100 (Sigma-Aldrich) in 50 mM of phosphate buffer pH 9 at room temperature for 1 h with the agitation of 30 rpm.

2.6. Characterization of the Cellulase Enzyme

The enzyme characterization was done by measuring the enzyme activity and stability during incubation at different temperatures (30, 40, 50, 60, 70, 80, and 90°C), pH (4-10), and the addition of metal ions (KCl, $CaCl_2$, $MgCl_2$, $FeCl_3$), surfactant (SDS) and chelating agent (EDTA) solution in 10 mM concentration (Gaur and Tiwari 2015).

3. Results

3.1. Identification of Cellulose-degrading Microorganism

Conventional identification was carried out by growing isolate C12 on CMC agar in a petri dish and then observing the morphology of the growing colonies, visually. In addition, observations of cells were carried out under a microscope with a certain magnification and gram staining. It was observed that isolate C12 are rod-shaped and Gram-negative bacteria (Figure 1) which form whitish, undulate-circular and raised colony shape in agar plate (Figure 2A). Figure 2B shows the clear zone formation of isolate C12 on Congo-red stained medium, that indicate its cellulose-degrading capacity.

Molecular analysis of microorganisms by 16-sRNA that the sample isolate C12 can be classified into *Leclercia adecarboxylata* species because it is in the same branch of phylogenetic tree and shows a bootstrap value of 64% (Figure 3). This is also appropriate with the BLAST results which showed that isolate C12 and *Leclercia adecarboxylata* had a percentage identity value of 99.50%.

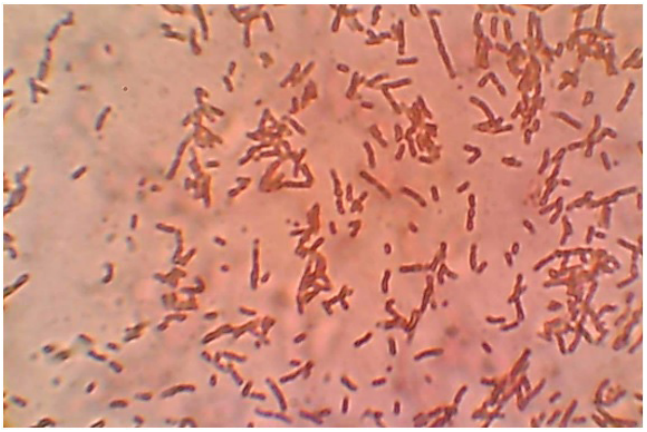
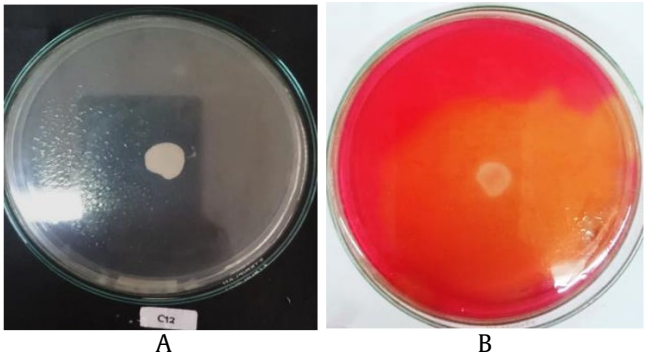


Figure 1. Isolate C12 cell's on microscope 100x magnification with Gram staining



A B

Figure 2. Isolate C12 growth colony appearance on CMC agar plate, before (A) and after (B) Congo Red staining

3.2. Effect of Incubation Time on Enzyme Activity and Bacterial Growth

Bacterial growth profile was obtained by plotting between incubation time and Optical Density (OD). OD can be used to measure the level of turbidity by using a certain wavelength, where the more turbid a liquid medium or solution shows the greater the number of bacterial masses. Meanwhile, the determination of the optimum time for cellulase enzyme production was carried out by testing the activity of the CMCase enzyme every day for 7 days of incubation. Afterward, daily enzyme activity observation data was plotted in a graph with incubation time as abscissa and CMCase activity as ordinate axis. Enzyme harvest time is determined by looking at the incubation time with the highest CMCase activity. Figure 4 shows the microbial

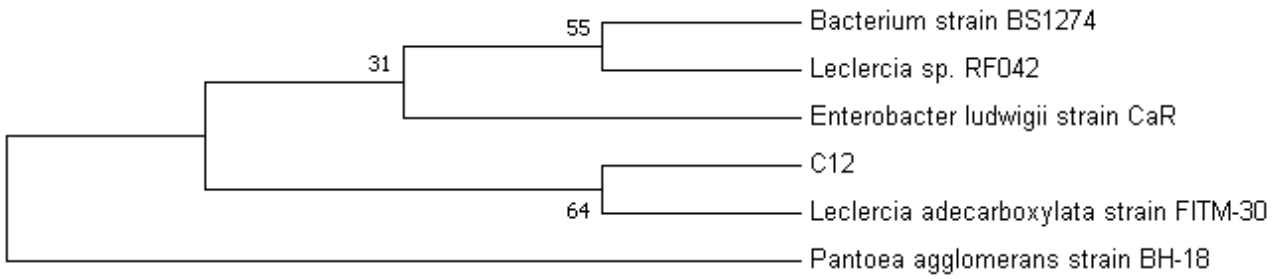


Figure 3. Phylogenetic tree of *Leclercia adecarboxylata* C12

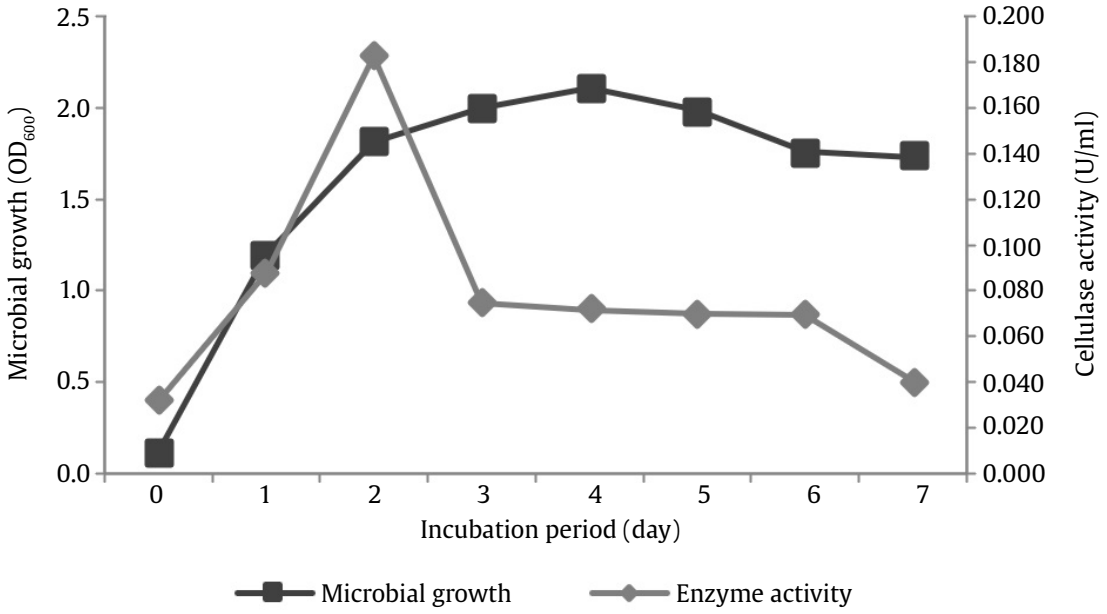


Figure 4. Effect of incubation period on microbial growth and enzyme activity of *Leclercia adecarboxylata* C12

growth and enzyme activity profile of *Leclercia adecarboxylata* C12.

3.3. Partial Purification of the Enzyme

Details for the cellulase purification are presented in Table 1. The crude enzyme extract contained 1053,538 mg protein showed 115,088 U/l in terms of total activity. In the final stage of dialysis the specific activity, yield and purification fold were 0.153 U/mg, 7.361% and 1.397, respectively.

3.4. Molecular Weight and Enzyme Activity in Polyacrylamide Gel

The purity of the enzyme was confirmed by the presence of two bands on SDS-PAGE with molecular weight 95.49 kDa and 81.28 kDa, respectively. Those bands reveal their cellulolytic ability during activity gel analysis with zymogram method by forming clear zone around the bands (Figure 5).

3.5. Effect of Temperature on Purified Enzyme Characteristic

The effect of temperature on *Leclercia adecarboxylata* C12 cellulase activity was observed over a broad range of temperature (30-90°C) with the optimal activity at 50°C and declined thereafter (Figure 6). Whereas, the stability of the enzyme was observed during 3 h incubation at 50°C. The enzyme maintained activity for 1 h at 50°C and began to decrease afterwards (Figure 7).

3.6. Effect of pH on Purified Enzyme Activity

The optimum pH for the cellulase from *Leclercia adecarboxylata* C12 was pH 9 (Figure 8). The cellulase enzyme stability test against pH showed that the cellulase of Leclercia adecarboxylata C12 increased in the pH range of 5-9 and was stable at pH 7 to 9 with relative activity above 90% (Figure 9).

3.7. Effect of Various Chemicals on Purified Enzyme Activity

The cellulase activity from *Leclercia adecarboxylata* C12 is known to be activated by the K^+, Ca^{2+}, Mg^{2+} and Fe^{3+} ions with the highest activity after the addition of Fe^{3+} ions reached 146.38±0.174%, as shown in Figure 10. Furthermore, the present of 10 mM of EDTA and SDS inhibited the cellulase activity until 44.06±0.01% and 65.74±0.49%, respectively.

Table 1. Purification of *Leclercia adecarboxylata* C12 cellulase

Purification steps	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	480	115,088	1,053,538	0.109	100.000	1.000
Ammonium sulphate 80%	300	63,830	488,100	0.131	55.462	1.197
Dialysis	55	8,472	55,521	0.153	7.361	1.397

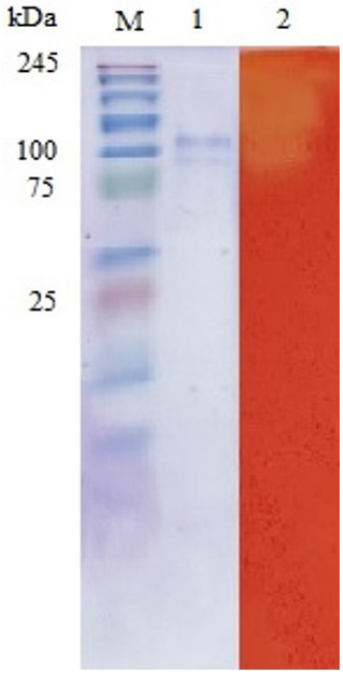


Figure 5. Zymogram analysis of *Leclercia adecarboxylata* C12 cellulase. Lanes: (M) MW protein standards, (1) comassie blue staining of purified enzyme, (2) clear zone formation of purified enzyme on Congo-red staining

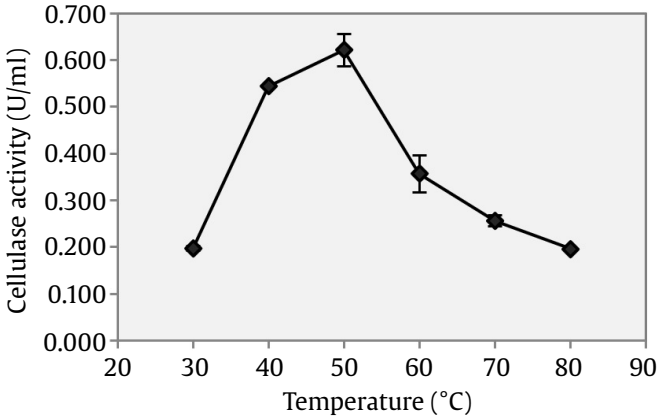


Figure 6. Effect of temperature on *Leclercia adecarboxylata* C12 cellulase activity

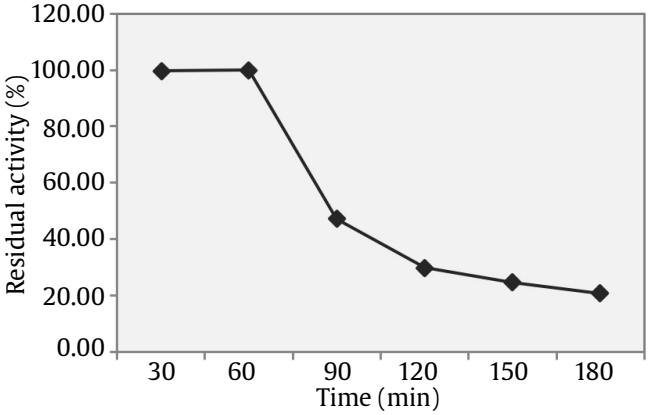


Figure 7. Thermal stability of *Leclercia adecarboxylata* C12 cellulase at 50°C

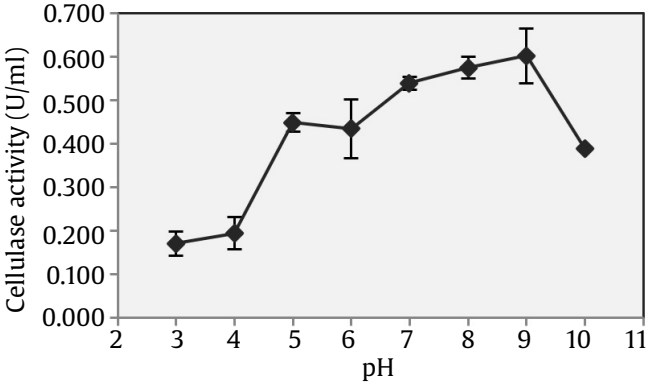


Figure 8. Effect of pH on *Leclercia adecarboxylata* C12 cellulase activity

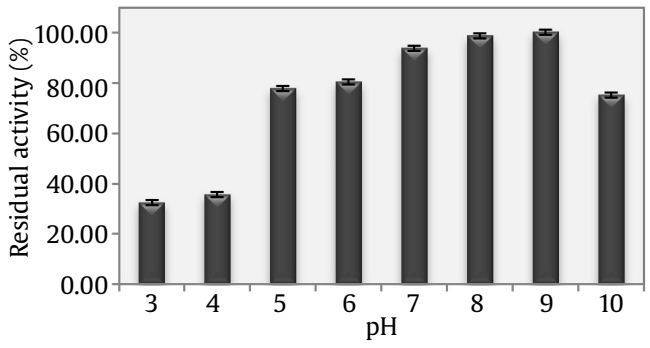


Figure 9. Enzyme stability in various pH value

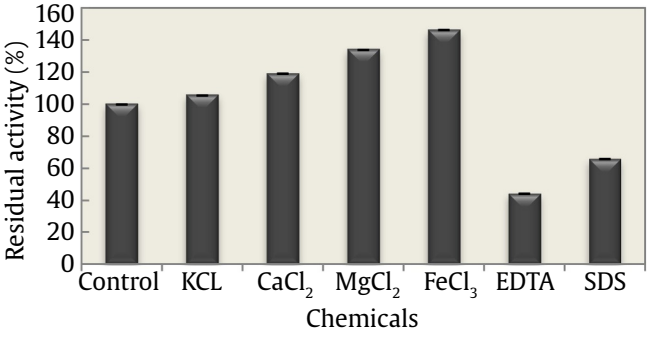


Figure 10. Effect of various chemicals on *Leclercia adecarboxylata* C12 cellulase activity

4. Discussion

This study shows the microbial diversity of cellulose-degrading microorganisms from Arabica coffee pulp. The best-obtained isolate was then identified as *Leclercia adecarboxylata* C12, a rod-shaped and Gram-negative bacterium. In this phase, the cellulase enzyme produced was limited, and its activity was relatively low, 0.088 U/ml. The initial phase to the accelerated growth phase is often called the lag phase, indicated by the line between the first and second days of bacterial growth. In this phase, the production of cellulase enzymes by bacteria increases, and its activity increases. The fastest rate of cell division is found in the logarithmic growth phase or exponential growth, with a short and constant generation time, the fastest cell growth, the most active cell metabolism, and the synthesis of cell materials is very fast. The most cellulase enzymes were produced in this phase, and the highest enzyme activity reached 0.183 U/ml. It was also reported that after one day of incubation, the OD_{600} value of Leclercia adecarboxylata culture came to 1.2. (Teng *et al.* 2019). The growth phase began to be inhibited on the 3rd day of incubation, in which the speed of cell division decreased, and the number of dead cells started to increase. This phase is called the stationary phase, where the number of dead cells increases until the number of living cells resulting from the division equals the number of dead cells as if there was zero growth. At the end of the 5th day of bacterial colony growth, the rate of cell death continued to increase while the rate of cell division was zero, so the number of living cells decreased rapidly like a geometric progression. Thus, the crude enzyme is produced by incubating Leclercia adecarboxylata culture for 48 hours.

Partial purification of *Leclercia adecarboxylata* C12 cellulase leads to an increase in the specific activity and a decrease in the total protein concentration. The reduction in total protein levels indicates the dialysis process was running well in removing impurity or non-enzyme proteins (Al-Kharousi *et al.* 2015; Gaur and Tiwari 2015). The specific activity of partially purified enzyme from *Leclercia adecarboxylata* C12 was known to be similar to cellulase from *Streptomyces* sp. (Malhotra *et al.* 2012) but lower than *Chaetomium* sp. (Al-Kharousi *et al.* 2015). The molecular weight analysis of the purified enzyme by electrophoresis results in appearance of two bands that show cellulolytic activity on zymogram analysis. Those bands indicate the cleavage of protein during the purification process, since the enzyme has a sequence for protease site or else it is a dimer.

The optimum temperature of *Leclercia adecarboxylata* C12 cellulase was similar to *Stachybotyrs* sp. and *Trichosporon* sp., which showed maximum activity at 50°C (Amouri and Gargouri 2006; Touijer *et al.* 2019). At the optimum temperature, the enzymatic reaction was the fastest, and the enzyme activity was the maximum since the collision between the enzyme and the substrate is very effective so that the formation of the enzyme-substrate complex becomes easier and the product formed increases (Baharuddin *et al.* 2014). Additionally, the viscosity of the CMC solution decreases as the incubation temperature rises. As a result, cellulase enzymes may have easier access to the substrate (Yusak 2004).

The thermostability of *Leclercia adecarboxylata* C12 cellulase decreased with increasing incubation time. It can be caused by releasing ligands or proteins that protect against the enzyme's active site (Ladeira *et al.* 2015). This is in accordance with the cellulase enzyme from *Stachybotrys* sp., which is optimum at 50°C and its relative activity decreases after 60 minutes of incubation and decreases with increasing temperature (Amouri and Gargouri 2006). Meanwhile, the optimum endoglucanase enzyme from *Bursaphelenchus xylophilus* at 50°C decreased its relative activity after 4 hours of incubation. The decrease became more drastic with increasing temperature (Zhang and Zhang 2013).

The pH value can influence cellulase activity, and changes affect the ionization of amino acid side groups in the pH of the medium. Maintaining the active enzyme's tertiary and quaternary structure

will impact the hydrogen bonds formed between functional groups and the conformation of the enzyme and substrate (Oyeleke *et al.* 2012). The cellulase enzyme will show the highest activity in hydrolyzing the substrate at optimum pH conditions. By the data shown in Figure 9, the optimum pH for the cellulase from *Leclercia adecarboxylata* C12 was pH 9. It was similar to the reported cellulase activity from *Bacillus* sp. WBS3, *Bacillus subtilis* AS3 and *Bacillus* sp. KSM-S237 that is respectively isolated from cow's dung, grass, and soil sample, then inoculated on CMC agar plates with a similar method as in this research (Acharya and Chaudhary 2012; Deka *et al.* 2011; Hakamada *et al.* 1997). The stability of *Leclercia adecarboxylata* C12 cellulase was in accordance with cellulase enzyme *Bacillus* sp. KSM-S237 that was stable above 80% at pH 5-10 (Hakamada *et al.* 1997), and *Streptomyces cellulase* retained relative activity above 60% at pH 5-9 (Jang and Chen 2003).

The cellulase activity from *Leclercia adecarboxylata* C12 was observed to be activated by K^{+}, Ca^{2+}, Mg^{2+} and Fe^{3+} ions with the highest activity after the addition of Fe^{3+} ion. It is in accordance with the cellulase from Bacillus vallismortis RG-07 (Gaur and Tiwari 2015), but contrary to the results of *Bursaphelenchus xylophilus* celullase that decreased the residual activity until 19.7 ± 2.2 after the addition of Fe^{3+} ions (Zhang *et al.* 2013). Furthermore, EDTA and SDS inhibits the cellulase activity since EDTA can bind with the –SH group with different degree interaction and subsequently inhibit the activity. These phenomenons suggested that the enzyme's active site contains –the SH group. SDS inhibits enzyme activity by triggering aggregation and causing denaturation of the enzyme.

The thermostability and alkali tolerance of the purified enzyme is useful for industrial applications, such as detergent making, textile, pulp, and paper production. However, this unique property of *Leclercia adecarboxylata* C12 purified cellulase has no relation with the properties of coffee-pulp waste as the source of cellulose-degrading bacteria isolation. Coffee-pulp waste was used as the source of bacteria isolation since it has a high content of cellulose, which leads to a higher possibility of obtaining cellulose-degrading microbes (Bui 2014; Eida *et al.* 2012; Junqueira *et al.* 2019). At the same time, it would reduce the enzyme production cost, increase the economic value of agro-industrial waste and decrease land pollutants. This newly isolated cellulose-degrading bacterium and its cellulase properties from the coffee pulp was novel research that had never been published before. Nevertheless, the cellulose-degrading capacity of *Leclercia adecarboxylata* has been indicated by other researchers (Nogales *et al.* 2012; Yadav *et al.* 2012).

Acknowledgements

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References

Acharya, S., Chaudhary, A., 2012. Bioprospecting thermophiles for cellulase production: a review. *Brazilian Journal of Microbiology*. 43, 844-856. https://doi.org/10.1590/S1517-83822012000300001

Al-Kharousi, M.M., Sivakumar, N., Elshafie, A., 2015. Characterization of cellulase enzyme produced by *Chaetomium* sp. isolated from books and archives. *EurAsian Journal of BioSciences*. 9, 52-60.

Amouri, B., Gargouri, A., 2006. Characterization of a novel β-glucosidase from a *Stachybotrys* strain. *Biochemical Engineering Journal*, 32, 191-197. https://doi.org/10.1016/j.bej.2006.09.022

Baharuddin, M., Patong, abd rauf, Ahmad, A., Nafie, N., La, 2014. Pengaruh suhu dan pH terhadap hidrolisis CMC oleh enzim selulase dari isolat bakteri larva kupu-kupu cossus cossus. *Teknosains*. 8, 343-356.

Beilen, J.B. va., Li, Z., 2002. Enzyme technology: an overview. *Current Opinion in Biotechnology*. 13, 338-344. https://doi.org/10.1016/S0958-1669(02)00334-8

Bhat, M.K., 2000. Cellulases and related enzymes in biotechnology. *Biotechnology Advances*. 18, 355-383. https://doi.org/10.1016/S0734-9750(00)00041-0

[BPPT] Badan Pengkajian dan Penerapan Teknologi, 2015. Ciptakan Pabrik Enzim Pertama di Indonesia, BPPT Transfer Teknologi Produksi Enzim ke PT Petrosida Gresik. Available from: https://www.bppt.go.id/teknologi-agroindustri-dan-bioteknologi/. [Date accessed: 14 January 2020]

[BPS] Badan Pusat Statistik, 2018. Indonesian Coffee Statistic 2018. Available at: https://www.bps.go.id/publication/2019/12/06/b5e163624c20870bb3d6443a/statistik-kopi-indonesia-2018.html. [Date accessed: 14 January 2020]

Brahmanti, A.A., Wardani, A.K., Martati, E., 2021. Exploring cellulolytic microorganisms from coffee industry by-products and their enzyme properties. *In: IOP Conference Series: Earth and Environmental Science.* Malang: IOP Publishing, (in press). https://doi.org/10.1088/1755-1315/924/1/012075

Bui, H.B., 2014. Isolation of cellulolytic bacteria, including actinomycetes, from coffee exocarps in coffee-producing areas in Vietnam. *International Journal of Recycling of Organic Waste in Agriculture*. 3, 48. https://doi.org/10.1007/s40093-014-0048-0

Deka, D., Bhargavi, P., Sharma, A., Goyal, D., Jawed, M., Goyal, A., 2011. Enhancement of cellulase activity from a new strain of bacillus subtilis by medium optimization and analysis with various cellulosic substrates. *Enzyme Research*. 2011, 1-8. https://doi.org/10.4061/2011/151656

Eida, M.F., Nagaoka, T., Wasaki, J., Kouno, K., 2012. Isolation and characterization of cellulose-decomposing bacteria inhabiting sawdust and coffee residue composts. *Microbes and Environments*. 27, 226-233. https://doi.org/10.1264/jsme2.ME11299

Elango, R., Divakaran, J., 2009. Microbial consortium for effective composting of coffee pulp waste by enzymatic activities. *Global Journal of Environmental Research*. 3, 92-95.

Feng, X., Dong, H., Yang, P., Yang, R., Lu, J., Lv, J., and Sheng, J., 2016. Culture-dependent and-independent methods to investigate the predominant microorganisms associated with wet processed coffee. *Current Microbiology*. 73, 190-195. https://doi.org/10.1007/s00284-016-1047-3

Gaur, R., Tiwari, S., 2015. Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. *BMC Biotechnology*. 15, 1-12. https://doi.org/10.1186/s12896-015-0129-9

Hakamada, Y., Koike, K., Yoshimatsu, T., Mori, H., Kobayashi, T., Ito, S., 1997. Thermostable alkaline cellulase from an alkaliphilic isolate, *Bacillus* sp. KSM-S237. *Extremophiles*. 1, 151-156. https://doi.org/10.1007/s007920050028

Islam, F., Roy, N., 2018. Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses. *BMC Research Notes*. 11, 1-6. https://doi.org/10.1186/s13104-018-3558-4

Jang, H.Der., Chen, K.S., 2003. Production and characterization of thermostable cellulases from *Streptomyces* transformant T3-1. *World Journal of Microbiology and Biotechnology*. 19, 263-268. https://doi.org/10.1023/A:1023641806194

Jaramillo, P.M.D., Gomes, H.A.R., Monclaro, A. V., Silva, C.O.G., Filho, E.X.F., 2015. Lignocellulose-degrading enzymes an overview of global market. In: Gupta, V.K., Mach, R.L., Sreenivasaprasad, S (Eds.). *Fungal Biomolecules*. New York: John Wiley and Sons, Ltd, pp. 75-85. https://doi.org/10.1002/9781118958308.ch6

Junqueira, A.C.O., de Melo Pereira, G. V., Coral Medina, J.D., Alvear, M.C.R., Rosero, R., de Carvalho Neto, D.P., Enríquez, H.G., Soccol, C.R., 2019. First description of bacterial and fungal communities in Colombian coffee beans fermentation analysed using Illumina-based amplicon sequencing. *Scientific Reports*. 9, 1-10. https://doi.org/10.1038/s41598-019-45002-8

Kuhad, R.C., Gupta, R., Singh, A., 2011. Microbial cellulases and their industrial applications. *Enzyme Research*. 2011, 1-10. https://doi.org/10.4061/2011/280696

Ladeira, S.A., Cruz, E., Delatorre, A.B., Barbosa, J.B., and Martins, M.L.L., 2015. Cellulase production by thermophilic *Bacillus* sp. SMIA-2 and its detergent compatibility. *Electronic Journal of Biotechnology*. 18, 110-115. https://doi.org/10.1016/j.ejbt.2014.12.008

Laemmli, U.K., 1970. Cleavage of structural proteins during the sssembly of the head of bacteriophage T4. *Nature*. 227, 680-685. https://doi.org/10.1038/227680a0

Lee, Y.J., Kim, B.K., Lee, B.H., Jo, K.I., Lee, N.K., Chung, C.H., Lee, Y.C., Lee, J.W., 2008. Purification and characterization of cellulase produced by *Bacillus amyoliquefaciens* DL-3 utilizing rice hull. *Bioresource Technology*. 99, 378-386. https://doi.org/10.1016/j.biortech.2006.12.013

Malhotra, J., Anand, S., Jindal, S., Rajagopal, R., Lal, R., 2012. *Acinetobacter indicus* sp. nov., isolated from a hexachlorocyclohexane dump site. *International Journal of Systematic and Evolutionary Microbiology*. 62, 2883-2890. https://doi.org/10.1099/ijs.0.037721-0

Miller, G.L., Blum, R., Glennon, W.E., Burton, A.L., 1960. Measurement of carboxymethylcellulase activity. *Analytical Biochemistry*. 1, 127-132. https://doi.org/10.1016/0003-2697(60)90004-X

Nogales, J., Concannon, M., Hall, M., 2012. Factors affecting the microbial digestion of an industrial seaweed-based residue. *Journal of Applied Phycology*. 24, 565-574. https://doi.org/10.1007/s10811-012-9805-5

Oyeleke, S., Oyewole, O., Egwim, E., Dauda, B., Ibeh, E., 2012. Cellulase and pectinase production potentials of *Aspergillus niger* isolated from Corn Cob. *Bayero Journal of Pure and Applied Sciences*, 5, 78-83. https://doi.org/10.4314/bajopas.v5i1.15

Pandey, A., Soccol, C.R., Nigam, P., Brand, D., Mohan, R., Roussos, S., 2000. Biotechnological potential of coffee pulp and coffee husk for bioprocesses. *Biochemical Engineering Journal*. 6, 153-162. https://doi.org/10.1016/S1369-703X(00)00084-X

Phuong, D.V., Quoc, L.P.T., Tan, P.V., Duy, L.N.D., 2019. Production of bioethanol from Robusta coffee pulp (*Coffea robusta* L.) in Vietnam. *Foods and Raw Materials*. 7, 10-17. https://doi.org/10.21603/2308-4057-2019-1-10-17

Silva, C.F., Batista, L.R., Abreu, L.M., Dias, E.S., Schwan, R.F., 2008. Succession of bacterial and fungal communities during natural coffee (*Coffea arabica*) fermentation. *Food Microbiology*. 25, 951-957. https://doi.org/10.1016/j.fm.2008.07.003

Teng, Z., Shao, W., Zhang, K., Huo, Y., Zhu, J., Li, M., 2019. Pb biosorption by *Leclercia adecarboxylata*: protective and immobilized mechanisms of extracellular polymeric substances. *Chemical Engineering Journal*. 375, 122113. https://doi.org/10.1016/j.cej.2019.122113

Touijer, H., Benchemsi, N., Ettayebi, M., Janati Idrissi, A., Chaouni, B., Bekkari, H., 2019. Thermostable cellulases from the yeast *Trichosporon* sp. *Enzyme Research*, 2019, 1-6. https://doi.org/10.1155/2019/2790414

Ulloa Rojas, J.B., Verreth, J.A.J., Amato, S., Huisman, E.A., 2003. Biological treatments affect the chemical composition of coffee pulp. *Bioresource Technology*. 89, 267-274. https://doi.org/10.1016/S0960-8524(03)00070-1

Utami, A.P., Setyaningsih, R., Pangastuti, A., Lusi, S.S.A., 2019. Optimasi produksi enzim selulase dari jamur *Penicillium* sp. SLL06 yang di isolasi dari serasah daun salak (*Salacca edulis*). *Pros. Sem. Nas. Masy. Biodiv. Indon*. 5, 145-149.

Wen, Z., Liao, W., Chen, S., 2005. Production of cellulase by *Trichoderma reesei* from dairy manure. *Bioresource Technology*. 96, 491-499. https://doi.org/10.1016/j.biortech.2004.05.021

Yadav, K.K., Shariq, M., Maurya, S.K., Alam, M., Ahmad, K., 2012. Molecular characterization of cellulose degrading bacteria on the basis of 16S RNA. *Journal of Recent Advances in Applied Sciences*. 80, 80-92.

Yusak, Y., 2004. Pengaruh suhu dan pH buffer asetat terhadap hidrolisa CMC oleh enzim selulase dari ekstrak *Aspergillus niger* dalam media campuran onggok dan dedak. *Jurnal Sains Kimia*. 8, 35-37.

Zhang, L., Fan, Y., Zheng, H., Du, F., Zhang, K.Q., Huang, X., Wang, L., Zhang, M., Niu, Q., 2013. Isolation and characterization of a novel endoglucanase from a *Bursaphelenchus xylophilus* metagenomic library. *PLoS One*. 8, 1-8. https://doi.org/10.1371/journal.pone.0082437

Zhang, X., Zhang, Y.P., 2013. Production and aplications. In: Shang-Tian Yang, Hesham A. El-Enshasy, Nuttha Thongchul (Eds.). *Bioprocessing Technologies in Biorefinery for Sustainable Production of Fuels, Chemicals, and Polymers*. New York: John Wiley and Sons, Inc. pp. 131-146.