Enzymatic activity of bacteria isolated from the gut of *Cylindroiulus* sp.: future prospect for decomposing agent

Ni’matuzahroh1,2,3*, M Affandi1,2, Fatimah1,2, N Trikurniadewi1,2, A Z Abidin1,2, A M Khiftiyah1,2, S K Sari1,2, S N M M Ibrahim1,2, M Janna1, A R Masrurin1, and R L Makrifah1

1 Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya 60115, Indonesia
2 University-Center of Excellence-Research Center for Bio-Molecule Engineering, Universitas Airlangga, Surabaya, 60115, Indonesia
3 Faculty of Advanced Technology and Multidiscipline, Universitas Airlangga, Surabaya, 60115, Indonesia

*E-mail: nimatuzahroh@fst.unair.ac.id

Abstract. Millipede *Cylindroiulus* sp. is well known as decomposer invertebrate. Its ability to break down organic waste is related to microbiota in their gut. Gut bacteria are known to be able to produce enzymes which have the potential as an organic waste decomposing agent. This study aimed to obtain potential bacteria in producing hydrolytic enzymes from the gut of *Cylindroiulus* sp. and to evaluate their enzymatic activities. Gut bacteria were isolated from *Cylindroiulus* sp. living in the household organic solid waste composter. Furthermore, isolated bacteria were analyzed quantitatively for their amylase, cellulase, protease, whereas qualitatively for lipase activities using semi-selective media. In this research, nine bacterial isolates were obtained. Three isolates namely EKG A1, EKG A4, and EKG A5 were able to produce all of the enzymes and identified as *Bacillus* genera. The highest index of enzyme activity in amylase, cellulase, and protease were 2.13 ± 0.08, 2.56 ± 0.03, 4.49 ± 0.17, respectively. *Cylindroiulus* sp. gut bacteria were prospectively applied to decompose organic solid waste.

1. Introduction

Decomposition of solid waste including household waste is an effort to reduce waste in the environment. Several city dwellers in Surabaya, Indonesia have formed composters to recycle organic household waste into other useful products such as compost. More than ten years, researchers have observed the decomposition process that occurs naturally in a household composter. Regularly, organic solid waste such as cooked food scraps, fruit skins, paper, litter, and twigs are put into the composter. The decomposition process in the composter only takes a relatively short time. In the process of decomposition of household waste, the composition and diversity of decomposer animals play an important role in it. About 19% of the composition of decomposer animals in the composter is millipede *Cylindroiulus* sp. [1].

*Cylindroiulus* sp. has been known to be a soil animal that acts as a decomposer and is active in the soil nutrient cycle [2]. According to Stoev and Enghoff [3] millipede is estimated to consume around 10-15% of leaf litter. In addition, they were found to eat mushroom mycelium and sporocarps [2].
These animals actively eat leaf litter, plant debris such as twigs, branches and rotten wood, as well as fruits and seeds that fall on the ground [4,5], so they play an important role in the process of plant decomposition, and rotting waste, soil formation, and biodegradation [6,7].

Millipedes is interesting to study because of its role as a saprophage animal and is widespread in various regions. The strongest suspicion of the ability of *Cylindroiulus* sp. in decomposing organic matter is the ability of enzymatic production in the digestive tract. The digestive process of litter does not only involve enzymes found in the digestive tract of the millipedes but also involves colonizing microorganisms in their digestive tract [6]. The millipede digestive tract contains high microbial diversity [8]. These microbes help in the digestion process of millipede by breaking down organic matter such as lignin, cellulose, and other plant recalcitrant polymers which are the main food source of millipede [6,9]. Bacteria from the Actinomycetes group such as *Cellulomonas, Nocardia* and Streptomycetes are known to colonize the millipede intestine and assist cellulolytic activity in the millipede digestive tract [10]. The intestine of millipede is a favorable environment for bacterial growth [6,11,12]. The secretion of amylase, cellulase, and other hydrolytic enzymes by the millipede intestinal wall illustrates the important role of the intestinal microbiota in the process of breaking down plant waste polymers [13].

Digestive enzymes such as cellulase, lipase, protease, and amylase which are derived from bacteria associated in the intestines of decomposer animals have the potential to overcome the problem of household organic waste buildup [1]. Although bacteria in the digestive tract of decomposers are potential as agents of organic waste decomposition, the research of potential local bacteria in producing hydrolytic enzymes from the intestine of *Cylindroiulus* sp. was not much done.

This study aims to obtain potential bacterial isolates that produce cellulase, amylase, protease and lipase hydrolysis enzymes from the intestine of decomposer *Cylindroiulus* sp. The results of this study are expected to add information about the ability of intestinal bacteria in decomposing household solid organic waste components. The ability of these bacteria can then be developed as a decomposition agent for handling urban household waste problems in the future.

2. **Method**

2.1. **Collection of Cylindroiulus sp.**
Sample *Cylindroiulus* sp. used in this study was millipede which commonly found in household organic waste composter. The decomposer animals and the compost were acclimatized in the laboratory for two weeks. Acclimatization was done by watering the compost every day to keep the compost moist and maintain the life of the decomposer animals.

2.2. **Isolation of Cylindroiulus sp. gut bacteria**
The digestive tract of a *Cylindroiulus* sp. was taken through dissecting. The surface of the digestive tract was sterilized following the method of Wynants et al [14] which had been modified. The digestive tract organs of *Cylindroiulus* sp. were washed with 10 mL of alcohol 70% for 15 seconds, then followed by rinsing in 10 mL of sterile distilled water twice for 15 seconds. The whole washing process was carried out using the vortex. Furthermore, the gut were mashed with NaCl 0.85% solution. The gut suspension was diluted serially. A total of 1 mL of the dilution was inoculated by pour plate method using Nutrient Agar (NA) media, then incubated for 24-48 hours at room temperature.

2.3. **Characterization of isolated bacteria**
Bacteria that grew on NA media will form colonies. The different bacterial colonies were transferred to a test tube to obtain a single isolate. Characterizations of bacteria were carried out through macroscopic observations of the of the bacterial colony morphology which included the shape, color, edges, consistency, and elevation and microscopic observation of bacterial cells using Gram and spore stainings. Characterization of bacteria was based on Bergey’s Manual of Determinative Bacteriology [15].
2.4. Enzymatic activities assay
The bacterial isolates obtained were tested for their enzymatic activity including amylolytic, cellulolytic, proteolytic, and lipolytic. The enzymatic activity tests were carried out by growing bacterial isolates on modified NA media. Modifications of NA media were done by adding 2% starch (w/v), 2% carboxymethyl cellulose (CMC) (w/v), 2% skim milk (w/v), and 1% glycerol (v/v) for amylolytic, cellulolytic, proteolytic, and lipolytic tests, respectively [16-19]. Hydrolytic enzymes activity was observed after 24 hours incubation at room temperature. The presence of enzyme activity was indicated by a clear zone formed around the bacterial colony. In the proteolytic enzyme activity test, the clear zone can be observed immediately after incubation [18], in the amylase enzyme, the test culture needed to be dripped with 2% iodine [20], the cellulase enzyme was dripped with congo red followed by washing using 1% NaCl [17], while the lipase enzyme was added with rhodamine, then observed under UV light [20]. Lipolytic enzyme activity was stated qualitatively, while amylase, protease, and cellulase enzyme activity were expressed quantitatively through the enzymatic activity index (EAI) calculated according to equation (1) [21,22].

\[
\text{EAI} = \frac{\text{clear zone diameter (mm)} + \text{colony diameter (mm)}}{\text{colony diameter (mm)}}
\]  

2.5. Bacterial identification
Three isolates with high enzymatic activity, consisted of isolates that able to show amylolytic, cellulolytic, proteolytic, and lipolytic activities, were identified based on their physiological characteristics. Physiological characterization was performed using the Microbact kit GNB 12A and 12B following manufacturer’s instruction (http://www.oxoid.com/pdf/uk/m-bact-gram-neg.pdf). The results of characterization were used to identify isolates by referring to Bergey’s Manual of Determinative Bacteriology [15]. The identification of bacteria was carried out to the level of the genus. The percentage of similarity between the isolates and the reference genus was calculated based on the Jaccard index [23].

3. Results and Discussion
3.1. Morphological and microscopical characteristics of isolated bacteria
The morphological characters of bacterial colonies isolated from the millipede’s gut were observed in 24-hour old bacterial cultures grown on Nutrient Agar media. A total of nine bacterial isolates showed different colony morphological characters (Table 1). Observation using light microscope revealed that all the isolates that were successfully grown had rod cell morphology and included Gram-positive bacteria, most of which were able to form endospores. Based on the research of Ambarish and Sridhar [24], it is shown that the bacteria that inhabit the millipede digestive tract can consist of Gram-positive and Gram-negative bacteria. This result is also in line with the research of Sridhar and Kadamannaya [25] which succeeded in isolating Gram-positive and Gram-negative bacteria from millipede stool pellet samples belonging to the alpha-proteobacteria, beta-proteobacteria, gamma-proteobacteria and bacilli groups. This indicates the richness of bacteria originating from the millipede digestive tract.
Table 1. Macroscopic and microscopic characters of *Cylindroiulus* sp. gut bacteria

| Isolate Code | Color | Macroscopic characters of colony | Microscopic characters of cell | Gram Staining | Existence of Spore |
|--------------|-------|----------------------------------|--------------------------------|---------------|-------------------|
| EKG A1       | Cream | Circular Entire Convex Opaque    | Rod +                          | +             |
| EKG A2       | White | Irregular Lobate Flat Opaque     | Rod +                          | +             |
| EKG A3       | White | Irregular Filiformis Flat Opaque | Rod +                          | +             |
| EKG A4       | White | Irregular Serrated Flat Opaque   | Rod +                          | +             |
| EKG A5       | White | Circular Entire Convex Translucent | Rod +                      | +             |
| EKG A6       | White | Irregular Serrated Flat Translucent | Rod +                      | +             |
| EKG A7       | Yellow | Circular Entire Convex Translucent | Coccoid +                  | -             |
| EKG A8       | Cream | Point Entire Flat Opaque         | Rod +                          | +             |
| EKG A9       | Cream | Circular Entire Convex Opaque    | Coccoid +                      | -             |

3.2. Enzymatic activities of gut bacteria

All bacterial isolates obtained were tested for their ability to produce enzymes that play a role in the decomposition process, namely the activity of amylase, cellulase, protease, and lipase enzymes. All isolates were able to show at least two different enzymatic activities. The enzymatic activity of each isolate is shown in Table 2. As many as 66.67% of bacterial isolates were able to show amylolytic and cellulolytic activity, 88.88% of bacterial isolates showed proteolytic activity, and all isolates showed lipolytic activity.

EKG A1 showed the highest amylase enzyme activity, indicated by a higher EAI value than other isolates, amounting to 2.13 ± 0.08. The highest cellulase and protease enzyme activity was obtained from EKG A5 isolates indicated by cellulase and protease EAI of 2.56 ± 0.03 and 4.49 ± 0.17 respectively. In the lipase enzyme activity, high activity was shown by EKG A3, EKG A4 and EKG A5. The results of the observation also showed that there were five isolates capable of producing four enzymes (multienzymes), namely EKG A1, EKG A4, EKG A5, EKG A6, and EKG A7 which showed the activity of amylase, cellulase, protease, and lipase enzymes.

Table 2. Enzymatic activity index (EAI) of isolated gut bacteria

| Isolate code | Amylase Index | Cellulase Index | Protease Index | Lipase Activity* |
|--------------|---------------|----------------|---------------|------------------|
| EKG A1       | 2.13 ± 0.08   | 1.72 ± 0.11    | 1.84 ± 0.02   | +                |
| EKG A2       | 0.00 ± 0.00   | 0.00 ± 0.00    | 1.31 ± 0.11   | +                |
| EKG A3       | 1.02 ± 0.16   | 0.00 ± 0.00    | 0.00 ± 0.00   | +++              |
| EKG A4       | 1.90 ± 0.00   | 0.96 ± 0.03    | 1.99 ± 0.14   | +++              |
| EKG A5       | 1.60 ± 0.08   | 2.56 ± 0.03    | 4.49 ± 0.17   | +++              |
| EKG A6       | 1.03 ± 0.03   | 1.15 ± 0.01    | 3.40 ± 0.24   | +                |
| EKG A7       | 1.18 ± 0.15   | 1.11 ± 0.07    | 1.70 ± 0.02   | +                |
| EKG A8       | 0.00 ± 0.00   | 0.00 ± 0.00    | 4.15 ± 0.32   | +                |
| EKG A9       | 0.00 ± 0.00   | 1.22 ± 0.01    | 2.72 ± 0.36   | +                |

* (-) not detected, (+) fluorescent colony, (++) fluorescent zone, (+++) fluorescent colony and zone

The presence of bacteria that are able to produce enzymes that play a role in the degradation of organic matter is not only found in *Cylindroiulus* sp., but in various other millipede groups. Among the Arthropods, millipede is the major detritus macroarthropod which contributes to the breakdown of organic matter [26]. They play an important role in the mechanical disintegration of organic matter, along with microbes doing the decomposition of starch and cellulose in the digestive tract [26,27] [28]. In the research conducted by Taylor [29], isolated bacteria from the intestines of millipede *Orthoporus ornatus* and *Comancheus* sp. were showed cellulase enzyme activity. The other research
conducted by Alagesan et al. [30] also showed that bacteria isolated from the intestine of millipede *Xenobolus carnifex* produced amylase, xylanase, cellulase, and protease enzymes.

In this study, the ability of the gut bacteria of *Cylindroiulus* sp. in producing hydrolytic enzymes that have a role in the decomposition of organic compounds carried out by *Cylindroiulus* sp. also involves the role of gut bacteria. Taylor [29] stated that decreasing of intestinal flora in millipede *Orthoporus ornatus* and *Comancheus* sp. caused the assimilation process of cellulose in the two millipede groups was also reduced.

### 3.3. Identification of gut bacteria

Based on the differences in macroscopic and microscopic morphological characters, physiological tests were carried out to determine the genus names of three potential isolates to produce multienzymes with high activity. The three isolates were EKG A1, EKG A4 and EKG A5. The physiological characters of the three isolates are shown in Table 3. According to the Jaccard index [23] the three isolates, namely EKG A1, EKG A4, and EKG A5 show similarities to the genus *Bacillus*. The genus *Bacillus* is known to have a symbiosis with millipede and inhabits the millipede hindgut [9,31]. In addition, according to Dhivya and Alagesan [6] *Bacillus thuringiensis* is also included in one of the microflora found in foregut, midgut, and hindgut milliped.

| Physiological characters | EKG A4 | EKG A5 | EKG A1 | Physiological characters | EKG A4 | EKG A5 | EKG A1 |
|--------------------------|--------|--------|--------|--------------------------|--------|--------|--------|
| Oxidase                  | +      | +      | +      | TDA                      | -      | -      | -      |
| Motility                 | -      | -      | -      | Gelatin                  | +      | +      | +      |
| Nitrate                  | +      | +      | -      | Malonate                 | -      | -      | -      |
| Lysine                   | -      | -      | -      | Inositol                 | -      | -      | -      |
| Ornithine                | -      | -      | -      | Sorbitol                 | -      | -      | -      |
| H$_2$S                   | -      | -      | -      | Rhamnose                 | -      | -      | -      |
| Glucose                  | -      | -      | -      | Sucrose                  | -      | -      | -      |
| Mannitol                 | -      | -      | -      | Lactose                  | -      | -      | -      |
| Xylose                   | -      | -      | -      | Arabinose                | -      | -      | -      |
| ONPG                     | -      | -      | +      | Adonitol                 | -      | -      | -      |
| Indole                   | -      | -      | -      | Raffinose                | -      | -      | -      |
| Urease                   | -      | -      | +      | Salicin                  | -      | -      | -      |
| VP                       | +      | +      | -      | Catalase                 | -      | +      | +      |
| Citrate                  | -      | -      | -      | Arginine                 | -      | -      | -      |

The genus *Bacillus* has been known to have many abilities, including producing various antimicrobial compounds, vitamins, and hydrolase enzymes such as amylase, protease, cellulase, and lipase which can degrade various types of polymers [31-33]. Bacilli, in particular, took initial and intermediate steps in polymer degradation such as cellulolytic activity, hemicellulolytic, and aromatic compounds [34]. In addition, its ability to form endospores makes *Bacillus* highly viable by being found in all environments including adaptive in the digestive tract environment which has different conditions from the external environment such as low pH, bile salts, anoxia, and the presence of other commensalism bacteria that are competitors to get it. places in the digestive tract [35,36].

Not many studies have revealed the ability of isolated *Bacillus* from the millipede digestive tract to produce various hydrolytic enzymes. Dhivya and Alagesan [6] stated that the activity of amylase and protease enzymes in *B. pumilis* and *B. subtilis* respectively were isolated from the gut of *Spinotarsus colosseus* and protease enzyme activity by *B. cereus* isolated from *Aulacobolus newtoni*. Thus, the results of this study provide new information that the isolated bacteria from the gut of *Cylindroiulus* sp. have the potential to be used as a decomposition agent in the future due to their ability to produce...
several enzymes at once. Therefore, various further studies to develop the potential isolated bacteria from *Cylindroïdulus* sp. must be done. Enzyme production and enzymatic assay on household waste are the objectives of the next research stage. Through this research and a series of further studies are expected to obtain the effective decomposition formula in managing solid organic waste in urban areas. Thus, municipal waste will be easily broken down and reduced rapidly.

4. Conclusions

Bacteria isolated from the gut of *Cylindroïdulus* sp. have the potential to produce enzymes such as amylase, protease, cellulase, and lipase. Potential bacterial isolates capable of producing multiple enzymes were EKG A1, EKG A4 and EKG A5. The highest index of enzyme activity in amylase, cellulase, and protease were 2.13 ± 0.08, 2.56 ± 0.03, 4.49 ± 0.17, respectively. The three isolates belonged to the *Bacillus* sp.

Acknowledgments

Author thanks the “Direktorat Riset dan Pengabdian Masyarakat, Deputi Bidang Penguatan Riset dan Teknologi/ Badan Riset dan Inovasi Nasional” who has funded research through “Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT)” Universitas Airlangga scheme, year 2020 with number contract: 820/UN3.14/PT/2020.

References

[1] Ni’matzahroh, Affandi M, Fatimah 2019 *Laporan Akhir Penelitian Dasar Unggulan Perguruan Tinggi Diversitas Mikroba Asosiatif pada Saluran Cerna Hewan Dekomposer Sampah Domestik : Upaya Produksi Enzim Potensial dan Formula Hewan Dekomposer dalam Pengolahan Sampah yang Efektif* Universitas Airlangga

[2] Semenyuk I I and Tiunov A V 2019 *European Journal of Soil Biology* 90 36-43

[3] Stoev P and Enghoff H 2008 *Steenstrupia* 29 47-66

[4] Suzuki Y, Grayston S J and Prescott C E 2013 *Soil Biology and Biochemistry* 57 116-123

[5] David J F and Gillon D 2002 *Pedobiologia* 46 pp 42-52

[6] Dhivyaa A and Alagesan P 2017 *International Journal of Microbiological Research* 8 19-24

[7] Hopkin H and Read H J 1992 *The Biology of Millipedes* (Oxford: Oxford University Press)

[8] König H and Varma A 2006 *Intestinal Microorganisms of Termites and Other Invertebrates* (Soil Biology vol 6) (Heidelberg: Springer)

[9] Byzov B A 2006 *Intestinal Microbiota of Millipedes Gut Microorganisms of Termites and Other Invertebrates (Soil Biology vol 6)* eds H König and A Varma (Heidelberg: Springer) pp 89-114

[10] Byzov B A, Chernjakovskaya T F, Zenova G M and Dobrovolskaya T G 1996 *Pedobiologia* 40 67–79

[11] Reyes V G and Tiedje J M 1976 *Pedobiologia* 16 67-74

[12] Hanlon R D G and Anderson J M 1980 *Soil Biology and Biochemistry* 12 255-261

[13] Ramanathan B 2013 *Diversity of millipedes and their effects on soil fertility of Alagar Hills of Tamil Nadu* Ph.D Thesis Madurai Kamaraj University, Madurai, India

[14] Wynants E, Frooninckx L, Crauwels S, Verreth C, De Smet J, Sandrock C, Wohlffahrt J, Van Schelt J, Depraetere S, Lievens B, Van Miert S, Claes J and Van Campenhout L 2019 *Microbial Ecology* 77 913-930

[15] Holt J G, Kreig N R, Sneath P H A, and Staley J T 1994 *Berger’s Manual of Determinative Bacteriology* 9th edition The William and Wilkins Co Baltimore Md USA

[16] Banerjee S and Ghosh K 2014 *Journal of Applied Ichthyology* 30 986-993

[17] Peristiwati, Natamihardja Y S and Herlini H 2018 *Journal of Physics: Conference Series* 1013 012173

[18] Bhowmik S, Islam S, Ahmed M M, Hossain M B and Hossain M A 2015 *Journal of Fisheries and Aquatic Science* 10 489-500
[19] Ni'matuzahroh, Trikurniadewi N, Ibrahim S N M M, Abidin A Z, Khiftiyah A M, Sari S K, Nuswantara E N, Nurmansyah F, Rahman M A R W, Maghfirah H L, Jannah M, Masrurin A R, Saidah L, Makrifah R L, Fatimah and Affandi M 2020 Ecology, Environment and Conservation 26 S123-S131

[20] Niyonzima F N and More S S 2014 Brazilian Journal of Microbiology 45 903-910

[21] Hankin L and Anagnostakis S 1977 Journal of General Microbiology 98 109-115

[22] Dantur K I, Enrique R, Welin B and Castagnaro A P 2015 AMB Express 5 15

[23] Real R and Vargas J M 1996. Systematic Biology 45 380-385

[24] Ambarish C N and Sridhar K R 2015 Journal of Agricultural Technology 11 637-648

[25] Sridhar K R and Kadamannaya B S 2009 Pill millipedes An overview Organic farming: methods, economics and structure eds Nelson M and Artamova I (USA: Nova Science Publishers Inc.) pp 76-77

[26] Edwards C A, Reichle D E and Crossley D A Jr 1968 Ecology 50 495-498

[27] Bano K 1979 Some Ecological Studies on the Millipede Jonespeltis splendidus in Relation to Soil Humification Ph.D. Thesis Bangalore University, Bangalore, India

[28] Sridhar K R and Ashwini K M 2016 Diversity, restoration and conservation of millipedes Biodiversity in India ed Pullaiah T (New Delhi: Regency Publications) 5 pp 1-38.

[29] Taylor E C 1982 Applied and Environmental Microbiology 44 281-291

[30] Alagesan P, Ashokkumar B, Muthukrishnan J and Gunasekaran P 2003 Indian Journal of Microbiology 43 111-113

[31] Gebhardt K, Schimana J, Muller J, Fiedler H P, Kallenborn H G, Holzenkämpfer M, Krastel P, Zeeck A, Vater J, Höltzel A, Schmid D G, Rheinheimer J and Dittner K 2002 FEMS Microbiology Letters 217 199-205

[32] Barros F F C, Simiqueli A P R, José de Andrade C and Pastore G M 2013 Biotechnology Research International 103960

[33] Elshaghabee F M F, Rokana N, Gulhane R D, Sharma C and Panwar H 2017 Frontiers in Microbiology 8 1490

[34] König H 2006 Journal of Applied Microbiology 101 620-627

[35] Bernardau M, Lehtinen M J, Forssten S D and P Nurminen 2017 Journal of Food Science and Technology 54 2570-2584

[36] Leser T D, Knarreborg A and Worm J 2007 Journal of Applied Microbiology 104 102