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Isolation and characterization of lactic acid bacteria from fecal pellets, coelomic fluid, and gastrointestinal tract of *Nypa* worm (*Namalycastis rhodochorde*) from West Kalimantan, Indonesia

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Abstract. *Yanti AH, Setyawati TR, Kurniatuhadi R. 2020. Isolation and characterization of lactic acid bacteria from fecal pellets, coelomic fluid, and gastrointestinal tract of* Nypa *worm* (Namalycastis rhodochorde) *from West Kalimantan, Indonesia. Biodiversitas 21: 4726-4731.* Lactic acid bacteria isolated from the intestinal tract as probiotic could be beneficial because they could trigger the growth of the host by enhancing digestibility, increasing their immune system, and inhibiting pathogenic bacteria through the enzymatic process or metabolites production. The purposes of this study were to identify and characterize lactic acid bacteria from fecal pellets, coelomic fluid, and gastrointestinal tract of *Nypa* worm *(Namalycastis rhodochorde)*. Bacterial isolation was carried out by the pour plate method on de Mann Rogosa Sharp Agar (MRSA). Lactic Acid Bacteria (LAB) isolates were purified and then physiologically characterized by some biochemical tests. Cellulolytic activities were carried out by detecting a clear zone formation on CMC-congo Red Agar. There were 20 isolates of lactic acid bacteria from fecal pellets, coelom fluid, and gastrointestinal tract of nypa worm. Ten isolates (50%) had cellulolytic activity on CMC-phenol red agar. Eight isolates were closely related to the genus *Lactobacillus*, while two isolates were closely related to the genus *Bacillus*. These cellulolytic bacteria could be developed further as probiotic in *Nypa* worm feed.

Keywords: *Bacillus*, cellulolytic activity, lactic acid bacteria, *Lactobacillus*, probiotic

INTRODUCTION

Aquaculture is a global industry that is not only important for the economic sector but also a source of high-quality food with potential nutrients from nature (Junardi 2014; Junardi et al. 2019). The use of feed containing Polychaeta for aquaculture has been carried out since it ensures the adequacy of nutrients because it contains several essential fatty acids for the development of the gonads (Costa et al. 2003). Application of *Nereis* sp. and probiotic in the shrimp feed could improve the biomass and health of the digestive tract of shrimp, thereby reducing the risk of developing viruses, bacteria, and other parasites (Pinon 2000; Costa et al. 2003). *Nypa* worm (*Namalycastis rhodochorde*) from *Nypa* mangrove waters in West Kalimantan, Indonesia, is one of a new species in Polychaeta (Glasby et al. 2007) that has the potential as a feed source for aquaculture. *Nypa* worms have protein content > 58% of the biomass (Junardi and Setyawati 2009).

Nypa worm cultivation on laboratory scale has been carried out to prevent over-exploitation of the worm in their natural habitat. The growth of *Nypa* worms in the laboratory was slower and more susceptible to parasites. It needs 3-4 months to reach 40 segments juvenile (Setyawati et al. 2015). Three components that influence the success of aquaculture are nutrition, digestibility, and disease prevention. According to Wiadnya et al. (2000), physiological aspects of digestion and feed are essential factors for the growth of aquaculture organisms. The slow growth of aquaculture organisms is also influenced by internal conditions, i.e., their low feed digestibility.

The digestibility of *Nypa* worms can be improved by administering probiotics (living microorganisms in the digestive tract) and prebiotics. A previous study by Yanti et al. (2017) showed that 50 bacteria isolates isolated from fecal pellets, coelomic fluid, gastrointestinal tract have proteolytic activity. Indigenous proteolytic bacteria have great potential to be developed as probiotics for aquaculture feed. Probiotic using indigenous microorganism has a higher probability of competitive exclusion due to adaptation to the same ecological niche (Lalloo et al. 2010). However, the potential of these bacteria must be investigated to determine their potency for improving growth and increasing the biomass of juveniles or adults of *Nypa* worm. Therefore, it is necessary to determine and select the potential cellulolytic bacteria as an indigenous probiotic in aquaculture, so that these microbes could be safely in feed formula for *Nypa* worm. The feed product containing probiotics are expected to be safe as energy sources to support the growth of aquaculture organism and a strategy to increase production in aquaculture.

MATERIALS AND METHODS

Study area

Sampling locations of *Nypa* worms (*Namalycastis rhodochorde*) were secondary mangrove areas located in the estuary of the Kakap River, Sungai Kakap Village, Kubu Raya District, West Kalimantan, Indonesia. The vegetation of secondary mangrove is dominated by *Nypa* or *nipah* trees (*Nypa fruticans* Wurmb.) as the primary habitat of *Nypa* worms.

Nypa worms that are collected should meet several conditions, i.e., fifty cm length, reddish in color, and intact body. Thirty uniform *Nypa* worms sample were maintained in the laboratory for the bacterial isolation process.

Preparation of growth medium for isolation

The process of isolation and characterization of lactic acid bacteria was carried out by culturing bacterial isolates on De Man Rogosa and Sharp (MRS) Agar (Meckmillipore). Weight 68.2 g of MRS agar, and then dissolved in 1L of distilled water followed by heating and then sterilizing in an autoclave at 121º C for 15 minutes.

Isolation and selection of lactic acid bacteria

Isolation of lactic acid bacteria (LAB) from fecal pellets, coelom fluids and the gastrointestinal tract of *Nypa* worms was carried out by pour plate and serial dilution methods. A sterile dissecting set was used to dissect the gastrointestinal tract of the *Nypa* worm. Ten grams of fecal pellets, coelom fluid, and gastrointestinal tract of *Nypa* worms were suspended in 90 ml of sterile saline solution and then agitated at a speed of 120 rpm for 30 minutes in a rotary shaker. Serial dilution was carried out by adding 1 ml of each suspension and mixing it with 9 ml of sterile saline buffer solution to a 10^{-5} dilution. One ml of three suspensions (10^{-3} - 10^{-5} dilution) were inoculated on MRS agar by pour plate method and incubated for 48 hours at 37ºC.

Morphologically different colony was purified on MRSA media. The purified-culture of each isolate was examined on MRSA using the quadrant streak method. Each purified culture was encoded based on sample type, namely NrLtF (fecal pellet), NrLtC (coelomic fluid), and NrLtG (gut). All bacterial isolates were preserved on MRS agar and stored at 4°C.

Screening of cellulase-produced lactic acid bacteria

The isolated lactic acid bacteria (LAB) were screened for their cellulolytic activity by determining the clear zone/ halo formed around the bacterial colonies on CMC-congo red agar (CMC 10 g/L; $MgSO_4.7H_2O$ 0.2 g/L; KNO_3 0.75 g/L; K_2HPO_4 0.5 g/L; $FeSO_4.7H_2O$ 0.02 g/L; $CaCl_2$ 0.04 g/L; yeast extract 2 g/L; D-glucose 1 g/L, agar 15 g/L). LAB cultures were inoculated by swabbing on the agar surface and forming a six mm-circle and then incubated at 37°C for 48 hours. After incubation, the formation of a clear zone on CMC-congo red agar indicates that bacteria secrete cellulase enzymes so that they can degrade cellulose. The clear zone and colony diameters were measured using a caliper. The cellulolytic index was calculated using the formula as follows (Ferbiyanto et al. 2015): Cellulolytic index: Diameter of the clear zone - Diameter of bacterial colony/ Diameter of bacterial colonies.

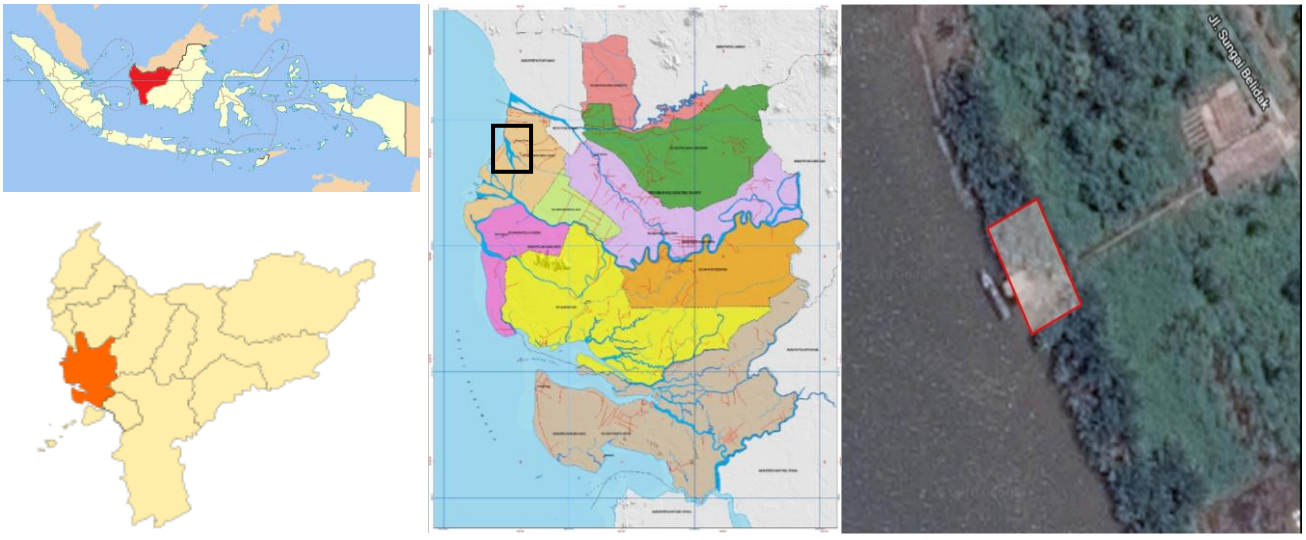

Figure 1. Sampling sites of *Nypa* worms (*Namalycastis rhodochorde*)in Sungai Kakap Village, Kubu Raya District, West Kalimantan, Indonesia

Morphological and biochemical characterization of selected-LAB

Morphological and biochemical characters of selected lactic acid bacteria were carried out by determining colony characteristics on the MRS agar medium and biochemical characteristics, and the ImVIC test. The observed morphological characters included cell shape by Gram staining, color, edge, and elevation of the colony. Biochemical characters were conducted based on Bergey's guidelines. Several biochemical tests include urease activity, ImVIC test, ornithine decarboxylase test, oxidase test, carbohydrate utilization production gas, and H_2S, gelatin liquefaction, oxidative fermentative metabolism, and catalase activity.

Determination of optimal pH and temperature for growth

Determination of optimum growth temperature and pH for growth was carried out by measuring the growth of lactic acid bacteria at various temperatures of 25º C, 30ºC, 35ºC, and pH values of 5, 7, 9 on the MRS broth medium. Bacterial suspensions adjusted to match the turbidity of 0.5 Mcfarland solution were inoculated into 100 ml MRS broth medium and incubated in an incubator shaker for 24 hours. After 24 hours, the turbidity of the bacterial suspensions was measured using a spectrophotometer with a wavelength of 600 nm. Optimum growth occurs in suspensions that have high optical density value.

Identification of selected lactic acid bacteria

All characteristics of selected lactic acid bacteria were identified based on in Bergeys Determinative of Bacteriology guidelines to determine the genus.

RESULTS AND DISCUSSION

Isolation and selection of lactic acid bacteria

The density of lactic acid bacteria from 30 samples of *Nypa* worms (*N. rhodochorde*) derived from fresh fecal pellets, coelomic fluid, gastrointestinal tract was presented in Table 1. The number of bacterial colonies isolated from fecal pellets was higher than coelomic fluid and gastrointestinal tract.

Twenty isolates of lactic acid bacteria were successfully purified, i.e., 11 isolates were derived from fecal pellets, six isolates from coelomic fluid, and three isolates from gastrointestinal tract. The selection of purified isolates was based on the different characters of bacterial colonies.

The ability of lactic acid bacterial isolates to break down carbohydrate macromolecules is determined by their ability to break down cellulose. It is indicated by the presence of a clear zone (halo) around the colony (Figure 1). There were 10 isolates (50%) of lactic acid bacteria that had cellulolytic activity (Figure 1) with varying diameter. NrLtG2 isolate had the largest halo diameter with a high relative index value (2.5) of the bacterial colony on the CMC-Phenol Red Agar Medium. The other bacterial isolates also had a very good index of cellulolytic activity, i.e. > 1(Table 2). Three bacteria isolates (NrLtC2, NrLtC4, and NrLtG2) had a relative index of cellulolytic activity > 2.

Ten isolates of lactic acid bacteria that have cellulolytic activity are Gram-positive, a chain of rod-shaped bacteria, or leaf-like mesophyll tissue (Figure 2.B). Eight isolates had similar colony characteristics, i.e. spherical, raised to convex, opaque, white, have no pigments (Figure 2.B). Two isolates (NrLtF7 and NrLtF9) had different colony characteristics with irregular colony forms and flat elevations (Figure 2.A).

The results of biochemical tests showed that seven isolates of lactic acid bacteria have weak catalase activity while the NrLtC2 bacterial isolate has no catalase activity. All isolates were facultatively anaerobic, oxidative and fermentative metabolism, did not produce gas and H_2S, positive methyl red test, but can not break down gelatin. Several biochemical tests including the citrate test, sucrose, and lactose test, cytochrome oxidase test showed varied (Table 3). All isolates can grow in a temperature range of 28-37ºC, with the optimum temperature for growth is 30ºC.

Based on the results of the morphological characterization of colonies, cells, and physiological characters, we obtained two different groups of bacteria. The first group consisted of eight bacterial isolates, namely NrLtF1, NrLtF2, NrLtF4, NrLtF5, NrLtF8, NrLtC2, NrLtC4, and NrLtG2 with the characters similar to the genus *Lactobacillus* such as 2-5 mm colonies on agar media, round and raised or convex colonies, entire, opaque, and without pigment on MRSA, rod-shaped (long rods), Gram-positive, non-sporing, facultative anaerobes, do not break down gelatin, catalase weak positive/negative and cytochrome oxidase negative. The second group consisted of two isolates (NrLtF7 and NrLtF9) with the characters similar to the genus *Bacillus* rod-shaped (long rods), Gram-positive, non-sporing, facultative anaerobe, catalase and cytochrome oxidase-positive.

Table 1. The density of lactic acid bacteria isolated from fecal pellets, coelomic fluid, and gut of *Nypa* worm (*Namalycastis rhodochorde*)

Number	Sample	Number of lactic acid bacteria (CFU/10 g)
1	Fecal pellets	2.5×10^2
2	Coelomic fluid	1.9×10^2
3	Gut	6.1×10^1

Table 2. The values of relative cellulolytic index values of lactic and bacteria isolated from fecal pellets, coelomic fluid, and gut of *Nypa* worm (*Namalycastis rhodochorde*)

Codes of isolates	Cellulolytic index (I_{cmc})
NrLtF 1	1.7
NrLtF 2	1.3
NrLtF 4	1.5
NrLtF 5	1.1
NrLtF 7	1.7
NrLtF 8	1.8
NrLtF 9	1.8
NrLtC 2	2.4
NrLtC 4	2.2
NrLtG 2	2.5

Discussion

The use of probiotics as an alternative to antibiotics has been recommended to be applied in the aquaculture to improve the immune system and optimize the digestibility of several organic macromolecules in aquaculture organisms. Caruffo et al. (2015) recommend the use of indigenous bacteria in the digestive tract be developed as probiotics because they are easier to adapt to the gastrointestinal host conditions so that it would be easy to reach optimal growth. Soccol et al. (2010) stated prolonged interactions of indigenous probiotic bacteria with hosts led to 'balanced relationships' in metabolic activity in both entities. In this study, we isolated microbes from the digestive tract of *Nypa* worms, and we obtained 58 isolates and 22 fungal isolates from fecal pellets, coelomic fluid, and gastrointestinal tract of *Nypa* worms. Twenty bacterial isolates were lactic acid bacteria (Yanti et al. 2017: Yanti et al. 2019).

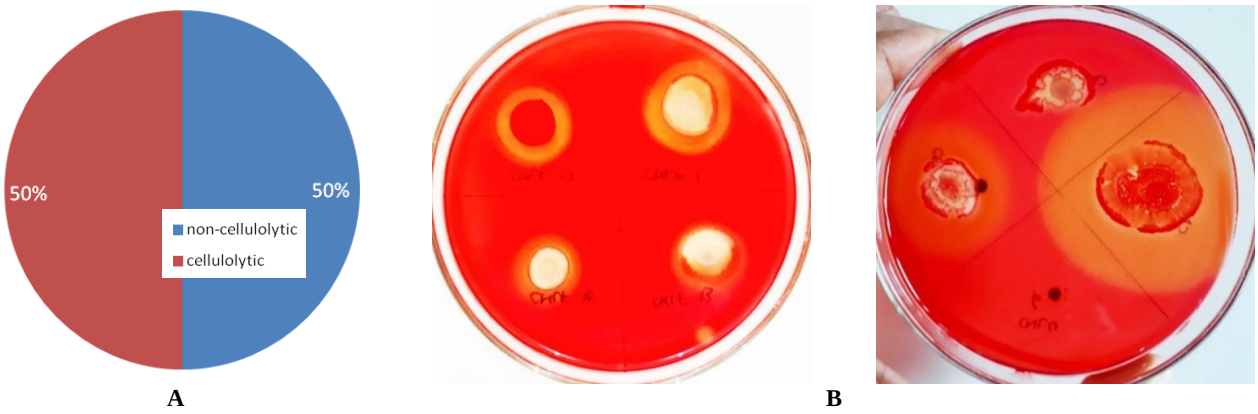

Figure 2. A. Percentage of cellulolytic and non-cellulolytic lactic acid bacteria isolated from *Nypa* worm (*Namalycastis rhodochorde*), B. The clear zone/halo formation on CMC Phenol Red Agar indicated cellulolytic activity of lactic acid bacteria

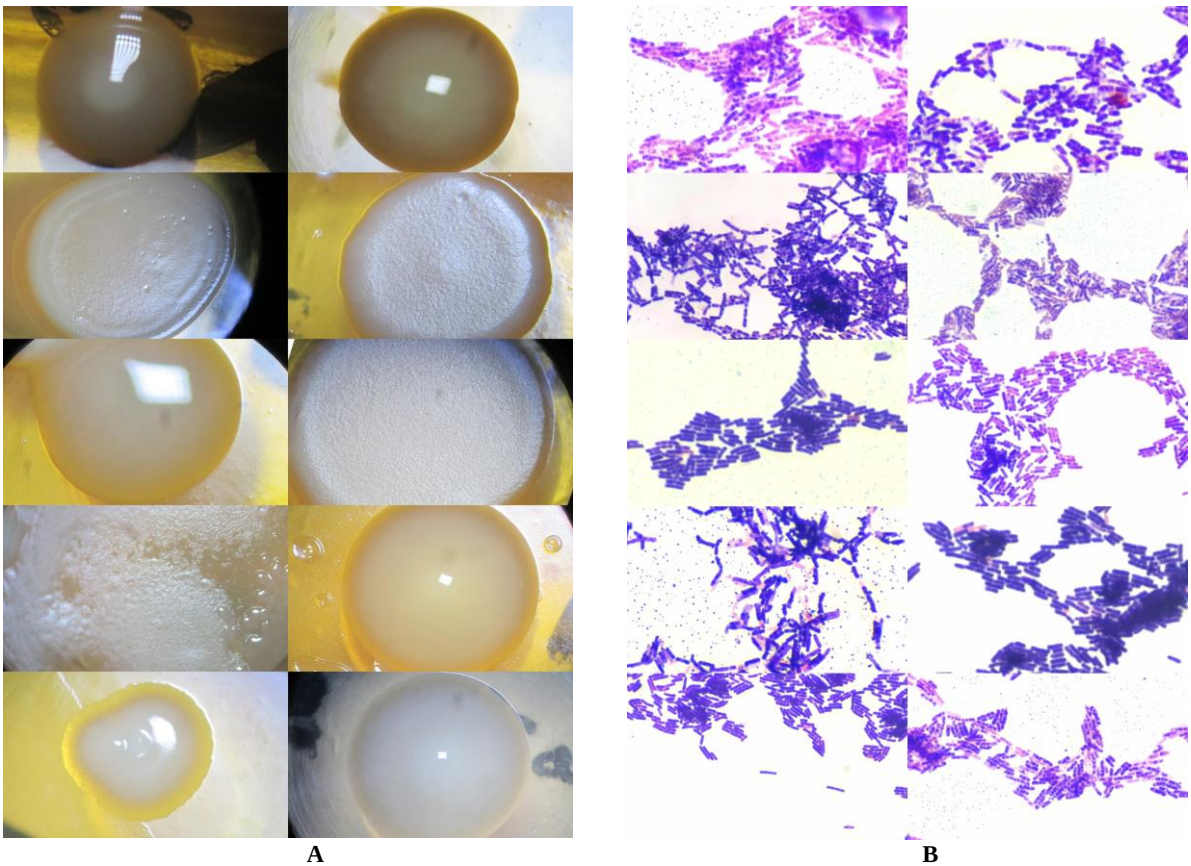

Figure 3. Characteristics of the bacterial colony on MRS agar (A); bacterial cell shape and formation (B) of cellulolytic-identified lactic acid bacteria isolated from fecal pellets and gastrointestinal cavity of *Nypa* worm (*Namalycastis rhodochorde*) from West Kalimantan, Indonesia

Table 3. Characteristics of selected lactic acid bacterial isolates from fecal pellets, coelomic fluid, and gastrointestinal tract of *Nypa* worm (*Namalycastis rhodochorde*)

Characters	Bacterial codes									
	NrLtF1	NrLtF2	NrLtF4	NrLtF5	NrLtF7	NrLtF8	NrLtF9	NrLtC2	NrLtC4	NrLtG2
Morphological characters:										
Cell shape	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Motility	-	-	-	-	-	-	-	-	-	-
Gram	+	+	+	+	+	+	+	+	+	+
Endospore	+	-	+	+	-	-	-	-	-	+
Cell formation	Chain	Chain	Chain	Chain	Chain	Chain	Chain	Chain	Chain	Chain
Form of colony	Round	Round	Round	Round	Iregular	Round	Round	Round	Round	Round
Elevation of colony	Raised	Raised	Convex	Convex	Flat	Convex	Raised	Raised	Convex	Convex
Color of the colony	White	White	White	White	White	White	White	White	White	White
Diameter of colony (mm)	8	4	5	5	20	4	19	5	4	4
Biochemical characters:										
Catalase	$+^{w}$	$+^{w}$	$+^{w}$	$+^{w}$	+	$+^{w}$	+	-	$+^{w}$	$+^{w}$
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-
Methyl red test	+	+	+	+	+	+	+	+	+	+
Citrate utilization test	-	-	+	+	-	-	-	-	-	-
Urease	+	+	+	+	+	+	+	+	+	+
Gas production	-	-	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-	-
Oxidative metabolism	+	+	+	+	+	+	+	+	+	+
Fermentative metabolism	+	+	+	+	+	+	+	+	+	+
Oxidase reaction	-	-	-	-	+	-	+	-	-	-
Utilization of:										
Glucose	+	+	+	+	+	+	+	+	+	+
Lactose	-	+	+	-	+	+	+	-	-	+
Succrose	-	+	+	-	+	+	+	-	-	+
Optimum ph and temperature										
pH range for growth	5-7	5-7	5-7	5-7	5-7	5-7	5-7	5-7	5-7	5-7
Temperature range for growth	28-37	28-37	28-37	28-37	28-37	28-37	28-37	28-37	28-37	28-37
Optimal temperature	30	30	30	30	30	30	30	30	30	30
Genera on *Bergey's*	L	L	L	L	B	L	B	L	L	L

Note: +: positive; $+^{w}$: weakly positive; - : negative; L: *Lactobacillus,* B: *Bacillus*

Previous studies showed that microbes in the gastrointestinal tract of worm consist of fungi, bacteria, actinomycetes, and protozoa (Yanti et al. 2020; Yanti 2019; Kadam and Pathade 2017; Sruthy et al. 2013). Govindarajan (2015) stated that the presence and composition of microbes in the intestinal of worms are due to the availability of mucus and organic compounds derived from natural food and supported by optimum physical-chemical factors in the intestinal tract. Twenty lactic acid bacteria were detected to have cellulolytic properties (Figure 2) so that they can break down the cellulose as the main biomass derived from the litter of *Nypa* leaves. This activity reflects the association of lactic acid bacteria with the digestive activity of *Nypa* worm, especially the cellulose substrate as feed. Based on enumeration of lactic acid bacteria from fecal pellets and digestive tracts of *Nypa* worm, it showed that bacterial concentration ranged from 6.1×10^1 to 2.5×10^2 CFU/10 g of samples (Table 1.). The number of these bacteria could show an association between lactic acid bacteria and conditions in the digestive tract of *Nypa* worms. The results showed that the optimum growth of bacteria was 30°C. This is consistent with the temperature of the study sites.

Lactic acid bacteria in fecal pellets, coelomic fluid, dan gastrointestinal tract of *Nypa* worms showed the ability to degrade cellulose. The values of the relative cellulolytic index values (Table 2.) indicates a relationship between the type of substrate, feed, and the association of gastrointestinal microbes in *Nypa* worm. We had profiling cellulolytic activity from twenty isolates isolated from *Nypa* worms, it indicated 50% of the lactic acid bacteria isolates producing cellulolytic enzymes. Bacteria isolated in this study were dominated by cellulolytic bacteria (Figure 3). This illustrated the relationship between enzymatic activity and the feed of the *Nypa* worm that predominantly was *Nypa* leaf litter mainly composed of cellulose. It stimulates the presence of cellulolytic bacteria in *Nypa* worm habitat. As a composite feeder, they can enter the gastrointestinal through the ingestion process.

Rowland et al. (2018) stated that intestinal microbiota is the key to describe biochemical profiles of diet or feed, metabolic characteristics, and character of associations with the lack of cellulase-produced hosts. Swetha (2014) stated that the substrate or feed containing cellulose could stimulate the growth of bacteria that can degrade cellulose into cellobiose and glucose disaccharides. A study by Junardi and Setyawati (2009) showed that the living *Nypa*

worm (*N. rhodochorde*) contain high organic carbon that indicated the decomposition process of *Nypa* litter and accumulated in the *Nypa* worm habitat or *Nypa* mangrove sediment. Therefore, cellulolytic bacteria become dominant in the habitat and gastrovascular tract of *Nypa* worm. Yanti et al. (2017) successfully isolated 32 lactic acid bacteria from *Nypa* mangrove sediment where *Nypa* worm lives.

Lactobacillus and *Bacillus* are the genera of lactic acid-produced bacteria that are most commonly found in various digestive tracts of animals associated with cellulose-rich habitats. *Lactobacillus* and *Bacillus* were successfully isolated from the intestinal of giant African snail (*Achatina fulica*: Gastropods), earthworm (*Eudrilus eugeniae*: Oligochaeta), and cotton bollworm (*Helicoverpa armigera*: Insecta) (Mudasir et al. 2017; Shankar et al. 2011: Govindarajan and Prabaharan 2015; Mudasir et al. 2018). The results in this study showed that *Lactobacillus* is also found in the intestine, coelomic fluid, and fecal pellets of *Nypa* worm. Gastropods, insects, and worms are known to use the same food sources derived from plants that contain cellulose. Lynd et al. (2002) stated that the symbiotic bacteria from the gut of herbivore are considered to participate in the digestion of cellulose comprising the major part of the plants. In conclusion, twenty isolates lactic acid bacteria had been isolated from fecal pellets, coelomic fluid, and gastrointestinal tract of *Nypa* worm. Ten isolates showed cellulolytic activity on CMC-phenol red agar. Eight isolates were closely related to the genus *Lactobacillus*, while two isolates were closely related to the genus *Bacillus*. *Lactobacillus* could be developed further as a probiotic for *Nypa* worm feed.

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