Identification and Probiotic Potency Screening of Cellulolytic Bacilli Isolated from Fecal Pellets and Gastrointestinal Tract of Nypha Worm (*Namalycastis rhodochorde*), West Kalimantan, Indonesia
(Identifikasi dan Skrining Potensi Probiotik Basil Selulolitik Diisolasi dari Pelet Tinja dan Saluran Pencernaan Cacing Nipah (*Namalycastis rhodochorde*), Kalimantan Barat, Indonesia)

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ABSTRACT

The bacterial isolates NrLtF1, NrLtF4, NrLtF5, and NrLtG2 isolated from fecal pellets and gastrointestinal tract of nypha worms (*Namalycastis rhodochorde*) have cellulolytic, proteolytic activity and produce organic acids. The four isolates have the potency to be developed as probiotics in nypha worm cultivation feed. This study aims to determine the probiotics potency and identify the species of NrLtF1, NrLtF4, NrLtF5, and NrLtG2 isolate based on 16srDNA sequence. The probiotic potency was carried out by the acid tolerance assays on distilled water and 0.3% acid bile media, and the antimicrobial testing against *Escherichia coli* (MF exp21.12). Bacterial identification was carried out by sequencing of 16sDNA sequence based on GeneBank data. The results showed that the bacterial isolates of NrLtF1, NrLtF4, NrLtF5, and NrLtG2 were able to grow on 0.3% distilled water and acid bile media. However, only the NrLtF4 and NrLtF5 inhibited *E. coli* (MF exp21.12) with halo zones 30 mm and 18 mm, respectively. Blasting results of the 16srDNA sequences showed that the NrLtF1, NrLtF4, NrLtF5, and NrLtG2 were closely related to *Bacillus wiedmannii*, *Brevibacterium sediminis*, *Bacillus proteolyticus*, and *Bacillus paramycoides*. The nypha worm bacterial isolates have the potency to be developed as probiotics in nypha worm culture.

Keywords: *Bacillus*, *Brevibacterium*, nypha worm, probiotic

ABSTRAK

NrLtF1, NrLtF4, NrLtF5, dan NrLtG2 yang diisolasi dari pelet tinja dan saluran pencernaan nypha cacing (*Namalycastis rhodochorde*) memiliki aktivitas selulitik, proteolitik dan menghasilkan asam organik. Keempat isolat memiliki potensi untuk dikembangkan sebagai probiotik dalam pakan budidaya nypha cacing. Penelitian ini bertujuan untuk menentukan potensi probiotik dan mengidentifikasi spesies nrLtF1, NrLtF4, NrLtF5, dan NrLtG2 isolat berdasarkan urutan 16srDNA. Potensi probiotik dilakukan melalui tes toleransi asam pada air suling dan 0,3% media empedu asam, dan pengujian antimikroba terhadap *Escherichia coli* (MF exp21.12). Identifikasi bakteri dilakukan dengan mengurutkan urutan 16sDNA berdasarkan data Gene Bank. Hasil penelitian menunjukkan bahwa isolat bakteri NrLtF1, NrLtF4, NrLtF5, dan NrLtG2 mampu tumbuh pada 0,3% air suling dan media empedu asam. Namun, hanya NrLtF4 dan NrLtF5 yang menghambat *E. coli* (MF exp21.12) dengan zona halo masing-masing 30 mm dan 18 mm. Hasil analisis 16srDNA menunjukkan bahwa NrLtF1, NrLtF4, NrLtF5, dan NrLtG2 terkait erat dengan *Bacillus wiedmannii*, *Brevibacterium sediminis*, *Bacillus proteolyticus*, dan *Bacillus paramycoides*. Isolat bakteri pada cacing nipah tersebut memiliki potensi untuk dikembangkan sebagai probiotik dalam kultur cacing nipah.

Kata Kunci: *Bacillus*, *Brevibacterium*, cacing nipah, probiotik

INTRODUCTION

Nypha worm (*Namalycastis* sp.) a worms from the polychaeta class that are spread in Southeast Asia, such as Indonesia, Malaysia, Brunei Darussalam, Philippines, and Thailand. The potential for worms in Indonesia, especially

in West Kalimantan, has the potential to be economically developed as raw material for aquaculture feed. *Namalycastis rhodochorde* is an endemic species in West Kalimantan which has a protein content of more than 58% -78% (Junardi *et al.* 2010) and has been introduced to aquaculture businesses and coastal communities

as raw material for fish and shrimp feed (Junardi *et al.* 2019).

Utilization of nipah worms (*Namalycastis rhodochorde*) as raw material for high protein aquaculture feed poses ecological challenges. Excessive exploration of nipah worms can cause a decrease in its population in nature. Junardi *et al.* 2007 have initiated in vitro nipah worm culture. In vitro culture of nipah worms has been successfully carried out until it reaches the juvenile phase. However, culture by invitro has constraints in the speed of growth. Research by Setyawati *et al.* (2010) showed that the survival rate of nipah worm larvae in phase three stages is less than 20.77% per month and the growth of the number of body segments to reach 40 segments takes four months. This is very different from the growth of nipah worms in nature which is faster with a length of up to 2 meters in a short time. In addition, larvae and juvenile nipah worms are also very susceptible to disease and parasite attack.

The problem of growth in vitro is the basis for the premise that the growth of nipah worms is very dependent on the availability of nutrients, associated microbes, and the ability to inhibit the growth of parasites and pathogenic microbes. Associated microbial has become a very important study and research in studying the nutritional requirements of several animal associations. The intestinal association of animals such as termites, earthworms, silkworms against cellulolytic microbes is known to help the growth of these animals in the process of providing and absorbing nutrients and as probiotic microbes.

Previous research (Yanti *et al.* 2017) has succeeded in isolating bacteria suspected of producing organic acids and lactic acid from fecal pellets and gastrointestinal nipah worms. Some isolates are known to have cellulolytic and proteolytic abilities and belong to the group of gram-positive bacilli that can form endospores (Yanti *et al.* 2020). The research of Khalid *et al.* (2021) stated that bacteria from the *Bacillus* group have the potential to be developed as probiotic candidates in animal feed. The results of Zeng *et al.* (2021) also used *Bacillus* as a probiotic agent isolated from the gut of broilers and analyzed the immune response and growth

of these broilers. The results of the addition of *Bacillus* probiotics in the study of Zeng *et al.* have a positive effect on the growth and immune system of broilers.

Most bacteria from the genus *Bacillus* are harmless to animal cultivation. *Bacillus* sp. It is known to produce a variety of functional antibiotics such as secondary metabolites, bio-insecticides, enzymes, and lipopeptides, such as iturin, surfactin, fengisin, and bacteriocin (Amin *et al.* 2020). These important biological and commercial characteristics make them suitable candidates for use as probiotic bacteria, including in the cultivation of nipah worms. The important role of probiotics is based on their antagonistic or antimicrobial activity against enteropathogenic bacteria. This is generally the result of bacteriocin secretion by probiotic cultures or competitive metabolic interactions between probiotics and pathogens.

The ability of these bacteria to break down cellulose from the nipa palm substrate and found in the nipah worm's gastrointestinal tract has the potential to be developed as a probiotic feed for nipah worms. In addition to increasing the digestibility function of nipah worms against cellulose substrates, these bacteria are also expected to provide other functional values, namely inhibiting the development of pathogens for nipah worms. Therefore, this study aims to detect the ability of bacteria as probiotics and to determine the type of species molecularly. This is the basis for further research for the development of probiotic feed for nipah worm cultivation.

MATERIALS AND METHODS

The catalase test was carried out by dripping 3% hydrogen peroxide (H₂O₂) on a clean object glass. The culture is smeared on an object glass that has been dripped with hydrogen peroxide with a dropper. The suspension is mixed slowly using a loop, a positive result is indicated by the formation of air bubbles (Hadioetomo 1990; Cappucino & Sherman 2005).

The glass slide that had been cleaned with 70% alcohol was fixed over a Bunsen fire, smeared with bacterial isolate using an ose needle and then the smear was burned three times over a

Bunsen fire. Furthermore, the isolate was dripped with crystal violet and allowed to stand for 3 minutes, then rinsed with distilled water and dried. Bacterial slides are observed under a microscope.

Erlenmeyer was filled with sterile distilled water and then adjusted until the pH reached 2.5. Then the distilled water was sterilized by autoclave at 121°C for 20 minutes. After sterilization, the distilled water was left to cool and followed by inoculation of 1 ose of bacteria in the distilled water. Then incubated in a shaker bath for 6 hours (Bayane *et al.* 2010). After 6 hours, the bacterial cell density was measured using a microplate reader.

The 2% bile acid medium was weighed and diluted using the dilution formula. To make 0.3% bile acid media, you need 30 mL of 2% bile acid in 200 mL of 0.3% bile acid media. Then sterilized in an autoclave at 121°C for 20 minutes. After cold media, 1 ose of bacteria were inoculated in bile acid broth media and incubated for 6 hours (Bayane *et al.* 2010).

Antimicrobial test was carried out by testing the sample bacteria with pathogenic bacteria, namely *Escherichia coli* (MF exp21.12). *E. coli* bacteria (MF exp21.12) were inoculated in liquid media and incubated for 24 hours. Then it was grown using the swipe method on solid media and also grown samples of bacteria in the middle of the media that had been planted with *E. coli* bacteria (MF exp21.12) and incubated for 3 x 24 hours. Probiotic bacteria were characterized by the presence of a clear zone in the media around *E. coli* (MF exp21.12).

Identification of bacteria was conducted by analysis of 16S rRNA sequences. Genomic DNA of each selected bacteria was isolated by using the Ultrac Clean Microbial DNA Isolation Kit according to the manufacturer's instructions. Thus, 16S rRNA gene was amplified by PCR using two

general primers 63F (5'-CAGGCCTAACACA-TGCAAGTC-3') and 1867R (5'- GGGCGGWG-TGTACAAGGC-3'). Amplification was carried out by PCR (2720 Thermal cycler applied bio-system) in volume of 25 µl consisting of .5 µl 10x Kappa taq buffer, 5 µl of 1 mM dNTPs, 1 µl of 10 mM each primer, 0.1 µl of Kappa taq DNA polymerase, 1 µl of DNA templates and 14.4 µl of deionized water. First denaturation was at 95°C for 3 minutes, 30 cycles with denaturation at 95°C for 1 min, annealing at 56°C for 45 s, extension at 72°C for i min 30 s, and the final extension for 4 min at 72°C. The products were checked by 1% agarose gel electrophoresis and stained with EtBr. The amplified product were sequenced. The sequences of 16S rRNA genes were used as query to determine the genus and species of its closest prokaryotic relative using BLAST.

RESULTS

Detection of the characters of four bacteria suspected of producing lactic acid isolated from the gastrointestinal tract of nipah worms (*Namalycastis rhodochorde*) showed similarities in all the characters analyzed again. The results of the observation of the characters of the four bacteria have a rod shape with consistent endospores, gram-positive groups, can perform 3% hydrogen peroxide breakdown activity, and are unable to degrade calcium carbonate on Glucose Yeast Peptone (GY) agar media (Table 1).

The four gram-positive rod-shaped bacteria are known to be able to grow and adapt to probiotic testing which includes a 6-hour acid tolerance test and a 0.3% bile acid test. The four bacteria were detected spectrophotometrically and grew again when streaked on the surface of GYP agar media.

Table 1. Characterization of cell shape, catalase activity, and degradation of CaCO₃ compounds by cellulolytic bacteria isolated from the gastrointestinal tract of nypa worm.

Isolate code	Characters				
	Cell form	Gram staining	Sporulation	Catalase activity	CaCO ₃ degradation
NrLtF1	Bacil	Positive	Center	Positive	Negative
NrLtF4	Bacil	Positive	Center	Positive	Negative
NrLtF5	Bacil	Positive	Center	Positive	Negative
NrLtG2	Bacil	Positive	Center	Positive	Negative

NrLtG2 bacteria are bacteria with a good tolerance value in the acid tolerance test for 6 hours in a medium without nutrients with a pH of 2.5 with an OD value: 0.062, while in the bile acid test 3% NrLtF1 bacteria are bacteria with a good tolerance value with an OD value of 0.181. Although the bacteria NrLtG2 and NrLtF1 had the best growth OD values in both acid tolerance tests, they did not have activity to inhibit *E. coli* using either test methods A or B. NrLtF4 and NrLtF5 bacteria are bacteria that have the potential to be developed as probiotic bacteria. In addition to these two bacteria being able to grow and adapt to the acid resistance test and 0.3% bile acid test, both bacteria were also able to inhibit the growth of *Escherichia coli* bacteria. NrLtF 4 bacteria had the largest clear zone diameter compared to the other three isolates, with an inhibitory diameter of up to 30 mm when using test method B (Table 2).

The measurement of the diameter of the clear zone showed that method A had a lower test effectiveness than test method B. Although the pattern of the two bacteria was the same, namely, NrLtF4 and NrLtF5 were able to inhibit the growth of *Escherichia coli*, the diameter of the inhibition zone formed was different in the two test methods. The clear zone diameter value of method B is larger than that of method A (Figure 1).

The results of the isolation of the 16sDNA gene showed that the four bacteria had the same DNA band weight of 1300 bp. The DNA bands of NrLtF1, NrLtF4, and NrLtF5 were well isolated, which was marked by the formation of clear DNA bands during electrophoresis, while the DNA bands of NrLtG2 isolates were not as bright as the other three isolates.

The results of the BLAST analysis showed that isolates NrLtF1, NrLtF4, NrLtF5, and NrLtG2

were known to have sequences that were similar to *Bacillus wiedmannii*, *Brevibacterium sediminis*, *Bacillus proteolyticus*, *Bacillus paramycoides* species. The isolates NrLtF1, NrLtF4, NrLtF5, and NrLtG2 were not known to be new indigenous strains in the NCBI database system after the BLASTING process. It is known that the similarity reaches 99% with the bacterial strain code registered in the database.

DISCUSSION

The use of probiotics as an alternative to antibiotics has been recommended to be applied in aquaculture to improve the immune system and optimize the digestibility of some organic macromolecules in cultured organisms. Caruffo *et al.* (2015) recommended the use of indigenous bacteria in the digestive tract to be developed as probiotics because they are more adaptable to the conditions of the gastrointestinal host so that they can easily achieve optimal growth. Soccol *et al.* (2010) stated that prolonged interaction of native probiotic bacteria with the host led to a

Figure 1. Antimicrobial activity of NrLTF4 and NrLTF5 isolates using the A/Kirby Bauer modified test method (top) and 6 mm swab test method (bottom).

Table 2. Test results of the probiotic character of cellulolytic bacteria isolated from the gastrointestinal tract of nypa worm.

Isolate code	Probiotic potency test				
	Acid tolerance test	Culture on GYP	Acid bile 0.3% test	Antibacterial test A (d; mm)	Antibacterial test B (d;mm)
NrLtF1	moderate	grow	tolerant	0	0
NrLtF4	moderate	grow	moderate	7	30
NrLtF5	moderate	grow	moderate	7	18
NrLtG2	moderate	grow	tolerant	0	0

'balanced relationship' in metabolic activity in both entities. Previous studies have conducted isolation of microbes from the digestive tract of nipah worms. The results of the isolation obtained 58 isolates and 22 isolates of fungi from fecal pellets, coelomic fluid, and the digestive tract of nypha worms (*N. rhodochorde*). Twenty bacterial isolates are bacteria that are thought to produce organic acids and lactic acid from their metabolic processes (Yanti *et al.* 2017; 2019; 2020).

Lactobacilli group bacteria are a group of bacteria that are commonly known to be able to produce organic acids in the form of lactic acid from their fermentative catabolism. The results of the repeated detection of four gastrointestinal isolates of nipah worms that were able to grow on MRS Agar media and produce organic acids showed that these bacteria belonged to the non-Lactobacilli group (Table 1). Previous studies have reported groups of non-Lactobacilli bacteria as organic acid and lactic acid producing bacteria. Research by Prasirtsak *et al.* (2013) succeeded in isolating bacteria capable of producing lactic acid from the *Paenibacillus*, *Bacillus*, and *Brevibacterium* groups from soil and leaf litter. The *Bacillus* group was also

reported by Tanasupawat and Komagata in 2001. Bacteria isolated from MRS Agar medium from the gastrointestinal tract of nipah worms (*N. rhodochorde*) were also identified from the genera *Bacillus* and *Brevibacterium* (Table 3). The results of this study strengthen existing research that lactic acid-producing bacteria do not only come from the Lactobacilli group. The Bacilli group, especially the genus *Bacillus* and *Brevibacterium*, has the potential to be developed as probiotic bacteria.

The probiotic potential of an isolate can be determined by several tests carried out related to its application to an organism. This is related to the early niche of the ancestral LAB theory proposed by Morelli *et al.* (2012) that the ancestors of LAB/BAL adapted to soil and gut conditions from plant eaters or plant litter. Tests for resistance to acid and 0.3% bile acid solution which are characteristics of the digestive tract have been carried out on the three genera of *Bacillus* and *Brevibacterium* isolated from nypa worm (*N. rhodochorde*). The four bacteria were able to grow on acid and bile acid test media (Table 2). These four bacteria have the potential to be developed as probiotics for nipah worm aquaculture feed. Probiotics are currently not only related to *Lactobacillus*, *Leuconostoc*, and *Bifidobacterium*. FAO/WHO has expanded the potential use of various microorganisms such as Gram-positive and Gram-negative bacteria, bacteriophages, microalgae and yeasts. Therefore the definition of probiotics is a live organism, which is administered orally has some obvious health benefits, and is widely used for disease control in culture, especially in developing countries (Irianto & Austin 2002; Nayak 2010).

The ability to inhibit pathogens is also a consideration in screening potential probiotic bacteria. *Bacillus proteolyticus* (code NrLtF5) and *Brevibacterium sediminis* (code NrLtF4)

Figure 2. Visualization of DNA bands of NrLtF1, NrLtF4, NrLtF5, and NrLtG2 bacterial isolates

Table 3. DNA sequence character equations in Gene Bank data.

No.	Kode Isolat	Spesies (BLAST NCBI)	Similarities (%)
1	NrLtF1	<i>Bacillus wiedmannii</i> strain FSL W8-0169	99
2	NrLtF4	<i>Brevibacterium sediminis</i> strain CGMCC 1.15472	99
3	NrLtF5	<i>Bacillus proteolyticus</i> strain MCCC 1A00365	99
4	NrLtG2	<i>Bacillus paramycoides</i> strain MCCC 1A04098	99

are known to have the ability to inhibit *E. coli*. This ability provides added value for the two bacteria from the gastrointestinal tract of the nipah worm, which has the potential to be developed as probiotics. *Brevibacterium* isolated from the gastrointestinal tract of nipah worms showed the ability to inhibit *E. coli* to form an inhibition zone or halo of 30 mm. This antibiosis potential was higher than the ability of *Brevibacterium* isolated by Motta & Brandelli (2002) against *Listeria monocytogenes* which was only able to produce a 10 mm halo zone.

Antibiotic ability of *Brevibacterium* and *Bacillus* has been known through the process of forming bacteriocin and linocin M-18 compounds which are also able to inhibit the growth of pathogens such as *Listeria monocytogenes* and *S. typhi* (Valdes-Stauber & Scherer 1994; Kuta *et al.* 2009). Research by Larsen *et al.* (2013) showed that bacteria from *Bacillus* spp. also developed as a probiotic with antibiotic function for pig feed. Cutting (2011) stated that *Bacillus* can be used as a probiotic supplement in animal feed. Their thermal stability and ability to withstand gastric acid make them potential as animal feed additives. Although *Bacillus* is often considered a soil organism, this conception is misplaced and *Bacillus* should be considered as an intestinal commensal, this is due to some animals that are detritivores in soil, including the nipah worm. Denkova *et al.* (2017) stated that according to the definition of probiotics by WHO and probiotic researchers that potential probiotic candidate bacteria are bacteria that are resistant to acidity tests and are able to inhibit several pathogenic organisms including aquaculture organisms. This indicates that *Bacillus proteolyticus* (code NrLtF5) and *Brevibacterium sediminis* (code NrLtF4) can be selected as probiotic candidates and need to be tested in-vivo through feed formulations for aquaculture organisms.

CONCLUSION

NrLtF1, NrLtF4, NrLtF5, and NrLtG2 isolates were known to have sequences that were similar to *Bacillus wiedmannii*, *Brevibacterium sediminis*, *Bacillus proteolyticus*, and

Bacillus paramycoides species. The four bacteria have characters that characterize the character of potential probiotic candidates, namely being able to grow on acid test media and 0.3% bile media. Two bacterial isolates were known to be able to inhibit the *E. coli* test pathogens with inhibition zone diameters of 18 and 30 mm, respectively. Potential probiotic bacteria derived from nipah worms can be developed through prebiotic mapping to obtain optimal growth. Therefore, further research is needed on mapping and the effect of prebiotic compounds on the growth of potential probiotic bacteria from nipah worms (*N. rhodochorde*). This will support the formulation of nipah worm animal feed which involves probiotic bacteria as one of its components.

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AUTHOR CONTRIBUTIONS

TRS, as main contributor, AHY, and RK contributed to data collection, data analysis, and of this article.

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