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Research Article

Effect of Different Growing Media on the Growth of Cellulolytic Bacteria from *Oryctes rhinoceros* Larva

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ABSTRACT

Oryctes rhinoceros larvae are one of the sources of cellulose and hemicellulose degrading bacteria. Nutrition and energy sources together, with environmental factors have significant impacts on cellulolytic bacteria growth. Carboxymethyl Cellulose (CMC) media is a media often used to obtain cellulosic bacteria, while Nutrient Agar (NA) media is used for bacteria propagation. The use of NA for bacterial growth media is expensive. For the growth of *O. rhinoceros* symbiont cellulolytic bacteria, it is necessary to use alternative media that are cheap, easily available and contain sufficient nutrients to sustain bacterial growth. Soybeans, mackerel tuna, and jackfruit seeds contain high levels of protein and carbohydrate, which can be used as a nitrogen and carbon source for cellulolytic bacteria growth are easily available and cheap. This makes them excellent alternative for growth media materials. The aim of this research was to determine growth of symbiotic cellulolytic bacteria from *O. rhinoceros* on different alternative growth media. The research was conducted from January to May 2024 at the Plant Disease Laboratory, Faculty of Agriculture and Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara. The experimental method used a factorial Completely Randomized Design (CRD) with 2 factors and 3 replications for each treatment combinations. The results showed that alternative growth media were suitable to sustain cellulolytic bacteria. The best treatment was mackerel tuna flour media with an average number of colonies of 100.5×10^6 CFU/mL.

Keywords: alternative media; *Oryctes rhinoceros*; symbiont bacteria

INTRODUCTION

Symbiont bacteria are bacteria that live permanently on a host, and usually produce the same bioactive compounds as their host. Some bioactive compounds originating from symbionts are often structuraly similar to metabolites from their hosts (Paul *et al*., 2021). The symbiosis between bacteria and insects is to have a biological impact on its host, such as producing digestive enzymes for digesting food (Dillon & Dillon, 2004). Symbiont bacteria can be found in almost all insect digestive system, including the gut of *O. rhinoceros* larvae, *Bombix mori* larvae, *Holotrichia parallela* larvae, *Reticulitermes flavipes*, *Anopplophora glabripennis*, and *Attacus atlas* L. (Dini *et al*., 2018).

O. rhinoceros hindgut comtain bacterial symbionts. The molecular identification of 16S rRNA successfully isolated and identified six bacterial strains (Marheni *et al*., 2021). These bacteria, which are potentially involved in lignocellulose degradation, were identified as *Bacillus stratosphericus*, *B. siamensis*, *B. cereus*, *Haemophilus parainfluenzae*, *Achromobacter xylosoxidans*, and *Alcaligenes faecalis*. The mass of the hindgut is approximately 150% the mass of the midgut. The pH of the midgut varied considerably, averaging 6.67 ± 2.08, while the hindgut pH varied less drastically, averaging 6.92 ± 0.88. Neither section were consistently more or less acidic than the other (Shelomi & Chen, 2020).

Decomposition of palm oil empty fruit bunches can be accelerated by symbiont bacteria derived from *O. rhinoceros* larvae. Conversion of cellulose, hemicellulose, and lignin compounds in palm oil empty fruit bunches can be degraded into simpler

compounds by cellulose-producing symbiont bacteria (Hasibuan *et al*., 2021). Bacteria that produce cellulose are called cellulolytic bacteria. Furthermore, palm oil bunches decomposed by *B. stratosphericus* and *B. siamensis* from *O. rhinoceros* larvae can be used as prenursery growth media (Sitanggang, 2021).

To grow and study the properties of microorganisms, suitable medium to grow the microorganisms of interest areessential. Nutrient Agar (NA) is a frequently used medium, consisting of 0.8% protein and 1.2% agar. As interest on microbiological examinations increase, the demand of NA also increases. However, NA is expensive (IDR 500,000−1,520,000/500 g) causing a challenge for microbiological studies (Asri *et al*., 2019). Therefore, various alternative sources have been explored as a growing media.

Various sources of plant-based proteins, fats, and carbohydrates such as soybeans which contain 40−43% protein (Azizah & Antarti, 2019). Meanwhile, other animal protein sources such as, mackerel tuna, contains 26.46% protein (Hidayat *et al*., 2020). Proteins, carbohydrates and fats in soya and tuna are sources of nitrogen and carbon that can be used for bacterial metabolism.

In addition to protein, bacterial growth also requires carbon obtained from carbohydrates. An easy to find and use carbohydrate is jackfruit seeds which contain 36.7 g of carbohydrates (Lestari, 2016). The content contained in soybean, mackerel tuna, and jackfruit seeds can serve as alternative growth media, due to their nutritional content for bacteria and fungi growth.

The role of *O. rhinoceros* symbiont cellulolytic bacteria in composting organic materials and high cost of instant NA (Nutrient Agar) media have encouraged researchers to explore alternative material for growing media of symbiont cellulolytic bacteria that are cheap, accessible and contain sufficient nutrients to sustain bacterial growth. The aim of this research was to observe the growth of symbiotic cellulolytic bacteria from *O. rhinoceros* on several growing media.

MATERIALS AND METHODS

This research was conducted from January to May 2024 at the Plant Disease Laboratory, Faculty of Agriculture and the Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara.

Experimental Design

This study was conducted using a factorial Completely Randomized Design (CRD). Factor I was the species of cellulolytic bacterial symbiont from *O. rhinoceros*, namely *B. subtilis* and *B. cereus*. Factor II was the media type, namely NA media, soybean flour media, mackerel tuna flour media, and jackfruit seed flour media. These two factors resulted in 8 treatment combinations with 3 repetitions resulting in 24 experimental units.

Media Treatments

Mackerel tuna flour media. Media from mackerel tuna meat was conventionally produced. The mackerel tuna meat was steamed for 30 minutes, mashed and dried in the oven at 50 ºC until dry. The dried fish was ground using a blender and sieved with a 100 mesh Tyler sieve to obtain finer flour grains (Al-Ayubi *et al*., 2022). Flour is determined when the fineness was 100 mesh or more, while powder fineness is between 10−80 mesh. Five grams of flour were immersed in 100 mL of warm distilled water for 2−3 hours. Solution was then filtered using sterile filter paper and 1.5 g of agar and 0.5 g of NaCl and 2 g of dextrose were added. The mixture was then heated on a hot plate while stirring until it dissolved. Media was sterilized using an autoclave at 121 ºC for 15 minutes.

Soybean flour media. Soybeans were sorted to obtain whole and round, seeds and remove foreign objects and selected soybeans were then immersed in water for 8−10 h. Skin was removed from previously immersed seed and dried in an oven at 50 ºC for 24 hours. Soybeans were ground or finely blended to obtain soybean flour. Soybean flour was filtered using a 100 mesh Tyler sieve (Indrayati *et al*., 2021). Five grams of soybean flour were immersed in 100 mL of warm distilled water for 2−3 h, filtered using sterile filter paper, and mixed with 0.5 g of NaCl, 2 g of agar, and 2 g of dextrose and placed in an Erlenmeyer, heated and stirred until completely dissolved. The media was then sterilized in an autoclave at 121 ºC for 15 minutes. Approximately 15 mL of sterilized

media was placed into a sterile Petri dishes, cooled and stored until further testing.

Jackfruit seed flour media. Jackfruit seeds were sorted and boiled for 30 minutes, drained for 5 minutes, sun dried. The dried jackfruit seeds were ground and sieved using a 100 mesh tyler sieve to obtain a finer flour (Pandanwangi *et al.*, 2023). Three grams of jackfruit seed flour were taken and soaked in 30 mL of warm distilled water for 2−3 h. The solution was then added to 0.95 grams of agar and 0.6 grams of dextrose and then put into the Erlemenyer to be heated and mixed on a hot plate at 80 ºC for 10 minutes. The cooked media was then sterilized using an autoclave for 15 minutes at 121 ºC.

Isolation of Cellulolytic Bacteria from the Intestines of *Oryctes rhinoceros* Larvae

Bacteria were isolated from the gut of *O. rhinoceros* larvae by piercing the larval abdomen and opening it with sterile scissors. Tweezers were used to extract intestine. The digestive system was then crushed by tearing the outer part of the intestine with tweezers until intestinal contents erupted. Intestinal contents were placed into an Erlenmeyer tube containing 100 mL of sterile water. The Erlenmeyer tube was then shaken with a shaker at 100 rpm for 1 hour to obtain a suspension that is expected to contain bacterial isolate candidates. The suspension was then diluted by serial dilution, i.e. 1 ml of suspension was added to a test tube containing 9 ml of distilled water and the suspension was diluted to 10^{-6}, 10^{-7}, and 10^{-8}. The solution of 10^{-6}, 10^{-7}, and 10^{-8}, 0.1 mL was inoculated on Carboximethyl Cellulase (CMC) selective solid media, then incubated at 30 °C for 48 hours. Bacterial colonies that grew were purified by taking one bacterial colony and inoculated onto NA media.

Qualitative Selection of Symbiont Cellulolytic Bacteria *Oryctes rhinoceros*

Bacterial isolates for qualitative testing were obtained from bacterial isolates on NA media. Cellulosic bacterial isolates were obtained using an ose needle on CMC agar media.

The diameter of the clear zone and the diameter of the colonies formed were measured using a digital caliper horizontally and vertically. The cellulase activity test was determined using the cellulolytic index formed. The cellulolytic index is the ratio between the clear zone and the colony diameter. The greater the cellulose index formed, the greater the enzyme produced by the bacterial isolate (Mulyasari *et al.*, 2015). The cellulolytic index or cellulase activity index was calculated using the following formula (Kader & Omar, 1998):

$$Cellulolytic\ Index = \frac{DB - DK}{DK}$$

DB = Clear zone (mm); DK = Colony diameter (mm).

Cellulolytic index could be categorized as low if Cellulolytic Index value was less than one, medium if value is between 1−2, and high if index is more than 2 (Choi *et al.*, 2005). After obtaining the Cellulolytic Index value, two bacterial isolates with the highest Cellulolytic Index value were selected. Each isolate was then morphologically, physiologically, biochemically characterized to determine the genus of the selected bacterial isolate.

Bacterial Characterization and Identification

Character of morphology and physiology of cellulolytic bacteria. Morphological characterization was done by observing bacterial colony color, shape, height, and edge shapes. Morphological characterization of cellulolytic bacteria was carried out by observing cell shape and Gram staining of bacteria. Physiology characterization of bacteria was done using Starch Hydrolysis Test, Citrate Test, TSI Test, Gelatin Test and Catalase Test.

Identification of Bacterial 16S rDNA

DNA extraction. Several bacterial DNA extraction methods can be used for 16S rRNA sequencing. where each method depends on the source of the DNA sample and the level of desired purity. Currently, many kits are available for effective and timely extraction. DNA extraction in this study was performed using the Quick-DNA Magbead Plus Kit (Zymo Research, D4082) (B/72.1/IKP/009).

16S rRNA gene amplification. DNA amplification used My taqTM HS Red mix kit, 2x (Bioline, BIO-25048) (B/7.2.1/OKP/002). The extracted DNA was used to amplify an approximately 500 or 1,500 bp region of the 16S rRNA gene sequence using the Polymerase Chain Reaction (PCR).

Electrophoresis. PCR products were further separated using gel electrophoresis to obtain visual bans of targeted sequence from symbiont bacterial isolate samples. Making 1 µL of amplification products with 0.8% agarose gel soaked in TBE buffer containing ethidium bromide. Visualizations was performed on a UV-transilluminator and documented.

DNA sequencing. Bidirectional sequencing using the sanger method DNA sequencing by using capiliary electrophoresis (1 st BASE subcontact lab testing). Bioinformatics analyses were used to process sanger sequencing results (B/7.2.1/IKP/006). The sequencing results are then Basic Local Alignment Search Tool (BLAST) using an online server (www.ncbi.nlm.nih.gov) to determine similarity with existing accession within the database.

Phylogenetic tree construction. DNA sequence from *O. rhinoceros* larval symbiont bacteria were used to compare with other species that listed in GenBank. Sequences from accessions showing similarity were collected and aligned using the CLUSTAW. A phylogenetic tree was constructed using MEGA XI using the neighbour-joining method.

Number of Colonies

After incubation for 24 hours, colony numbers were counted using a Colony Counter. The number of colonies that grew could be calculated to be in the range of 30−300 colonies.

Data Analysis

The observational data were analyzed using ANOVA. Colony numbers were analyzed using ANOVA. If the ANOVA results were significantly different, and DNMRT (Duncan New Multiple Range Test) post-test was conducted at the 5% level.

RESULTS AND DISCUSSION

Selection of Cellulolytic Bacterial Symbionts of *Oryctes rhinoceros* Larvae from Empty Palm Oil Branches

Cellulolytic activity of *O. rhinoceros* symbiont bacteria was indicated by the formation of a clear zone on CMC media after treatment of 0.1% congo red dye. The clear zone formed is caused by the process of cellulose degradation by cellulolytic bacteria.

The ability of bacteria to hydrolyse cellulose is expressed by the Cellulolytic Index or Cellulolytic Activity Index. The Cellulolytic Index value is determined by the diameter of the clear zone formed and the diameter of the colony. Cellulolytic Index values in the medium category are found in isolates with codes AS 10 and AS 8 isolates showed medium Cellulolytic Index, while AS 1 and AS 5 showed low Cellulolytic Index (Table 1). Isolate AS 10 showed the highest Cellulolytic Index, 1.26, while isolate AS 1 had the lowest Cellulolytic Index value of 0.09.

Based on these Cellulolytic Index test results, 2 isolates showing the highest cellulose degradation were selected, namely AS 8 (P1) and AS 10 (P2), for morphological and physiological characterization and later biochemical testing.

Bacterial Identification Based on 16S rRNA

Bacterial identification based on 16s rRNA revealed that the P1 bacteria was *B. subtilis* and P2 bacteria was *B. cereus* (Figure 1).

Biochemical Test of *Oryctes rhinoceros* Larval Digestive System Cellulolytic Bacterial Symbionts

Cellulosic bacteria, *B. subtilis* and *B. cereus*, showed positive results in the starch hydrolysis test based on the clear zone formed on the colony after

Table 1. Quantitative cellulolytic index of *Oryctes rhinoceros* larval digestive system symbiont bacteria

No.	Isolate Code	Clear Zone Diameter (mm)	Colony Diameter (mm)	Cellulolytic Index	Category
1	AS 1	66.45	60.70	1.09	Low
2	AS 5	10.80	6.84	0.57	Low
3	AS 8 (P1)	34.20	17.05	1.00	Medium
4	AS 10 (P2)	42.55	18.80	1.26	Medium

dropping iodine solution (Table 2). In the citrate test, bacteria reacted negatively by not changing color from green to blue. In the gelatin test, positive results were marked by media turning into liquid after storage in the refrigerator. Cellulosic bacteria can react positively to catalase test indicated by the presence of oxygen bubbles after isolates were placed on a glass object treated with H_2O_2 solution. The TSI test results for *B. subtilis* showed red slant indicating alkaline condition, yellow butt indicating acidic conditions, negative gas indicated by no rise of media, and negative H_2S indicated by black media. *B. cereus* showed red slant indicating alkaline conditions, the butt was yellow indicating acidic conditions, negative gas indicated by no rise of media, and positive H_2S indicated by media changing into black.

Morphology and Physiology Characters of Cellulolytic Bacteria

Morphological and physiological characterization of cellulolytic bacterial symbiont colonies from *O. rhinoceros* larval digestive system was based on colony shape, colony edges, colony height, colony color, gram staining and cell shape of each bacterium.

B. subtilis bacteria colonies were irregularly shaped, had undulate colony edges, raised colony elevations, and white (Table 3 and Figure 2), *B. cereus* colonies were circular, the edge of the colonies were full (flat), colonies were convex, and white (Figure 2).

Although morphological characters showed differences, their physiological characterization showed similarities between isolates. *B. subtilis* and *B. cereus*

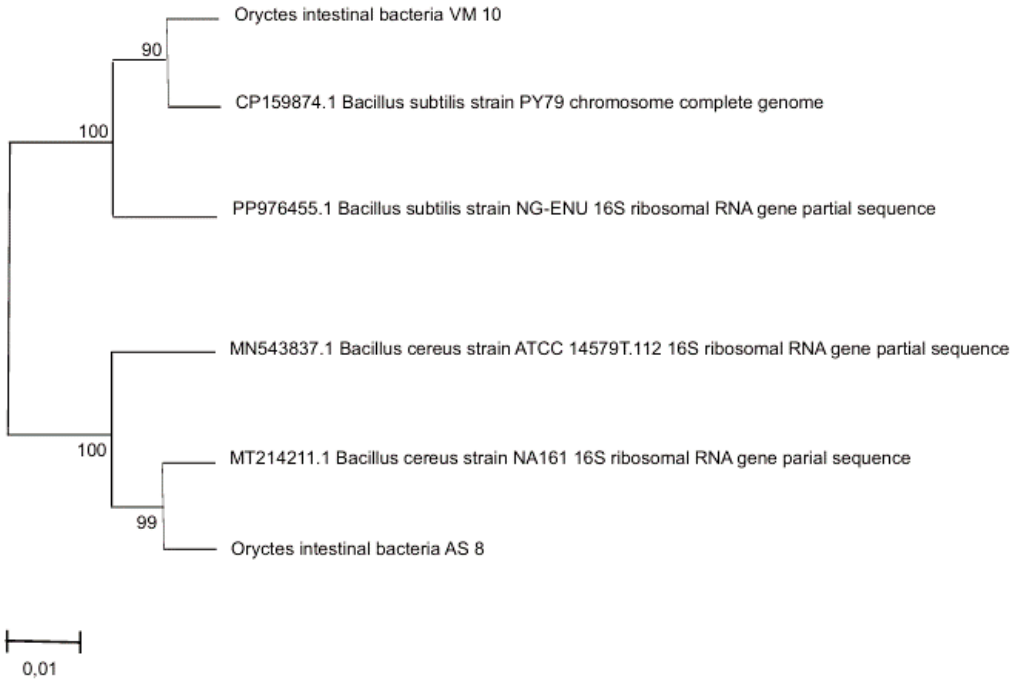

Figure 1. Phylogenic tree of the cellulolytic bacterial symbionts of *Oryctes rhinoceros* larval digestive system.

Table 2. Biochemical test results of cellulolytic bacterial symbionts from *Oryctes rhinoceros* larval digestive system

Biochemical Test	Types of Bacteria	
	Bacillus subtilis	*Bacillus cereus*
Starch Hydrolysis	Positive	Positive
Citric	Negative	Negative
Gelatin	Positive	Positive
Catalase	Positive	Positive
TSIA	A/K gas (-) H_2S (-)	A/K gas(-) H_2S(+)

Description : */* = Slant/butt, A = Red, K = Yellow

Table 3. Morphological and physiological characteristics of cellulolytic bacterial symbionts from *Oryctes rhinoceros* larval digestive systems

Morphological and Physiological Tests	Types of Bacteria	
	Bacillus subtilis	*Bacillus cereus*
Colony Form	Irreguler	Circular
Colony Edge	Undulate	Entire
Colony Elevation	Raised	Convex
Colony Color	White	White
Cell shape	Bacilli	Bacilli
Coloring Grams	Positive	Positive

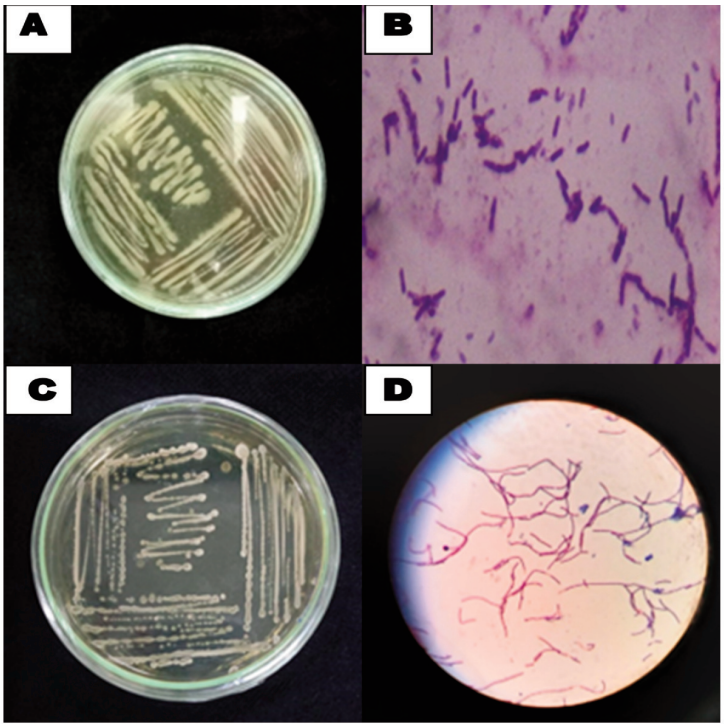

Figure 2. Macroscopic and microscopic forms of bacterial symbiont from *Oryctes rhinoceros* larval digestive system; *Bacillus subtilis* isolate colony (A), *Bacillus cereus* bacterial Gram stain (B), *B. subtilis* isolate colony (C), *B. cereus* bacterial Gram stain (D).

were rod-shaped cells (bacilli) and Gram-positive bacteria based on their purplish-blue staining (Figure 2).

Number of Colonies

The treatment types of media affected colony numbers where K2, K3, and K4 were significantly different from the control treatment (K1)(Table 4). However, colony numbers were not significantly influenced between bacteria based on statistical testing even though *B. cereus* (K2) grew better than *B. subtilis* (K1) based on number of colonies. The average colony number of *B. cereus* (P2) was 102.75 $\times 10^6$ CFU/mL and 97.75$\times$ 10^6 CFU/mL for *B. subtilis* (P1). This was influenced by the ability of each bacterium to grow on medium and environmental conditions. This was similar with Nafion *et al.* (2019) where different bacterial growth were caused by the different abilities of bacteria to reproduce depending on medium and nutrients in each medium.

Furthermore, type of media had effects colonies numbers. Colony numbers from soybean flour (K2), mackerel tuna flour (K3), and jackfruit seed meal (K4) were less than the control (K1). The average colony number were 157.33$\times 10^6$ CFU/mL from NA (K1), 100.5$\times 10^6$ CFU/mL from mackerel tuna (K3), 83$\times 10^6$ CFU/mL from soybeans (K2), and

Table 4. The effect of various alternative media on colony numbers (10^6 CFU/mL) of cellulolytic bacterial symbionts of *Oryctes rhinoceros* larval digestive system.

Types of Bacteria	Types of Media				Average
	K1	K2	K4	K5	
	..... Number of Bacterial Colonies (10^6 CFU/mL).....				
P1	153 $\times 10^6$	80.67 $\times 10^6$	98.67 $\times 10^6$	58.67 $\times 10^6$	97.75 $\times 10^6$
P2	161.67 $\times 10^6$	85.33 $\times 10^6$	102.33 $\times 10^6$	61.67 $\times 10^6$	102.75 $\times 10^6$
Average	157.33 $\times 10^6$a	83 $\times 10^6$c	100.5 $\times 10^6$b	60.17 $\times 10^6$d	

Description: Numbers followed by different letters in the same row are significantly different in Duncan's Multiple Range Test at α = 5% level; P1 = *Bacillus subtilis*, P2= *Bacillus cereus*, K1= Nutrient Agar media, K2= soybean flour media, K3= mackerel tuna flour media, K4= jackfruit seed flour media.

60.17×10^6 CFU/mL from jackfruit seeds (K4). This may be caused due to mackerel tuna, soybeans, and jackfruit seeds complex nutrients causing lower bacterial growth compared to nutrient agar media. NA media is an instant media that has been tested good for bacterial growth, so that the metabolic process of bacteria takes place optimally. This was similar to findings from Jawetz *et al.* (2005) where microorganisms growth slowed in media with complex nutrient content because microorganisms required longer time to break down simple components that can be absorbed by cells and used for cell synthesis and energy. This is also in line with Zamilah *et al.* (2020) where bacteria will grow faster when sufficient nutrients are available and will need to adjust to their environment and form enzymes to break down the substrate when these conditions are not optimum.

Colony number was not significantly impacted by the interaction between the bacteria species and media type. This condition is influenced by the nutritional requirements that each bacterial cell requires to reproduce in their environment and nutrient availability. This was in accordance with Hasibuan *et al.* (2022) where no significant interactions between the two treatments were detected due to one factor being more dominant or the interaction betwen two factors did not support each other for growth. This was also in accord with Fahruddin (2018) where cells grow rapidly only an environment that supports them. The ability to divide more quickly allows certain bacterial populations to adapt quickly to changes in the environment. Wartono and Priatno (2009) stated that bacterial growth in vitro is influenced by nutrient content, media pH, aeration, and incubation temperature. The optimum growth conditions vary between genus, species, and strains of bacteria.

Mackerel tuna flour (K3) showed the highest colony number among tested alternative growth media. This may be caused by its balance macro and micro nutrient content, especially its high protein which is a source of carbon, nitrogen, phosphorus, and sulfur. Parnanto *et al.* (2013) demonstrated that fish body consist of 75% oxygen, 10% hydrogen, 9.5% carbon, 2.5-3% nitrogen, 1.2−1.5% calcium, 0.6−0.8% phosphorus and approximately 0.3% of sulphur. Cilia *et al.* (2016) adds that mackerel tuna flour has a protein content of 64.31 (%), fat of 6.29 (%), water of 5.74 (%), ash of 10.30 (%), fiber of 2.57 (%) and other of 10.79 (%). Yustinah *et al.* (2016) added that microorganism cells contained carbon, chlorine, phosphorus and sulfur in a ratio of 100:10:1:1. These elements must be present in their food source for optimum microorganims growth.

Soybean flour media was the second-best media among artificial media for growing bacteria based on its colony numbers due to soybean flour nutritional content being quite high. The nutritional content of soybean flour, especially protein in soybeans, is quite high and the highest of all types of beans. Protein is a source of nitrogen and carbon for bacterial growth. In addition, soybeans also contain fats and carbohydrates which are carbon sources for bacterial metabolism. Basalamah *et al.* (2018) stated that protein compounds, both essential

and non-essential, consist of long chains of amino acids made up of the elements C, H, O, and N. Rahman *et al.* (2020) added that soybeans contain many nutritional values such as protein, fat, and vitamins. Soybeans can be used as a substitute for peptone in growth media.

Jackfruit seed flour demonstrated the lowest number of colonies. Jackfruit seed flour media contains less macro and micro nutrients than the other two types of alternative media. However, the carbohydrate content of jackfruit seed flour is the highest compared to other artificial media. Carbohydrates are a carbon source for bacterial growth. This is in line with Radji (2009), who stated that bacteria crave carbohydrates as their main source of metabolism. Almost half the dry weight of a bacterium is elemental carbon. Carbon can be found in carbohydrate, protein, and fat compounds. Therefore, carbohydrates are essential for bacterial growth. Materials that can be used as bacterial growth media are materials that are able to provide nutrients for bacterial growth.

CONCLUSION

Both symbiont bacteria isolate from *O. rhinoceros* larval digestive system showed different morphological characterization but similar in physiological characterization. *B. cereus*, which generated the most colonies on each media with an average of 102.75×10^{-6} CFU/mL. At an average of 100.5×10^{-6} CFU/mL, the tuna fishmeal media had the greatest number of colonies compared to other alternative media.

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