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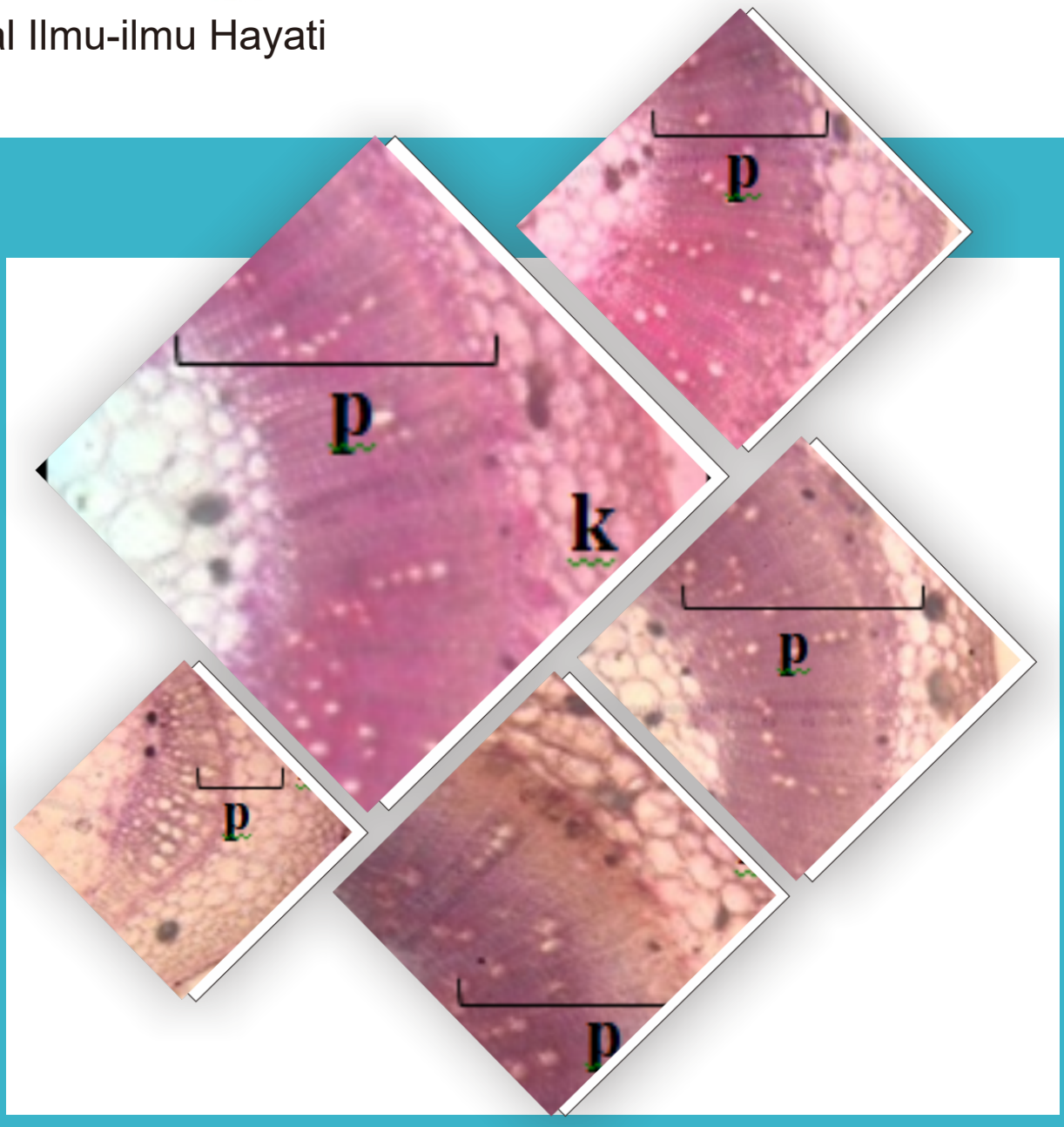

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Alamat (*Address*)
Pusat Penelitian Biologi-LIPI
Kompleks Cibinong Science Center (CSC-LIPI)
Jalan Raya Jakarta-Bogor KM 46,
Cibinong 16911, Bogor-Indonesia
Telepon (021) 8765066 - 8765067
Faksimili (021) 8765059
Email: berita.biologi@mail.lipi.go.id
jurnalberitabiologi@yahoo.co.id
jurnalberitabiologi@gmail.com

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CHARACTERIZATION OF SUPERNATANT EXTRACT AND VIABILITY OF *BACILLUS SUBTILIS* KM16 AND *PSEUDOMONAS* SPP. IN FISH FEED AS BIOCONTROL AGENTS AGAINST AQUACULTURE PATHOGENS

[Karakterisasi Ekstrak Supernatan dan Viabilitas *Bacillus subtilis* KM16 dan *Pseudomonas* spp., di Dalam Pakan Ikan Sebagai Agen Biokontrol terhadap Patogen Akuakultur]

Stella Magdalena[1*], Brenda Kristanti[2], Yogiara[3]

[1]Atma Jaya Catholic University of Indonesia, Faculty of Biotechnology, Department of Food Technology, BSD Campus, Banten, Indonesia
[2]Atma Jaya Catholic University of Indonesia, Faculty of Biotechnology, Department of Biotechnology, BSD Campus, Banten, Indonesia
[3]Atma Jaya Catholic University of Indonesia, Faculty of Biotechnology, Department of Magister of Biotechnology, Semanggi Campus, Jakarta, Indonesia
email : stella.magdalena@atmajaya.ac.id

ABSTRACT

The use of biocontrol agent in aquaculture is being adapted as an effective alternative to antibiotics which can lead to the elaboration of antibiotic-resistant bacteria and confer unpleasant impacts to aquatic organisms. Aquatic bacteria have been discovered as biocontrol agents and potential probiotic candidates to improve the health of aquatic organisms, feed efficiency, and disease resistance to aquaculture pathogens. However, local isolate has not intensively been explored and used to increase aquaculture sector productivity. Therefore, this research aimed to determine minimum inhibitory concentrations of their antibacterial compounds against aquaculture pathogens and to characterize aquatic bacteria by their viability in the feed. Four isolates from several aquatic environments in Indonesia (*Pseudomonas* sp. S1.1, *Pseudomonas* sp. S1.2, *Pseudomonas* sp. SL1.1, and *Bacillus subtilis* KM16) were used to characterize of antibacterial compound and to determine the viability in feed. Ethyl acetate extracts from all isolates showed better antibacaterial activity against *Aeromonas hydrophila* and *Vibrio vulnificus* than chloroform and dichloromethane extracts, in which ethyl acetate extract from *Bacillus subtilis* KM16 showed the strongest antibacterial activity. *Pseudomonas* spp. were more effective against *V. vulnificus* (40 mg/mL) and *Bacillus subtilis* KM16 was more effective against *A. hydrophila* (20 mg/mL), as proved by the minimum inhibitory concentrations of their ethyl acetate extracts. In this research, *Bacillus subtilis* KM16 had stable viability in feed than *Pseudomonas* sp. isolates.

Key words: Aquaculture, Antibacterial, *Bacillus*, Biocontrol, *Pseudomonas*

ABSTRAK

Agen biokontrol merupakan alternatif pengganti antibiotik yang efektif dalam budidaya akuakultur, dimana penggunaan antibiotik dapat menyebabkan perkembangan sifat resistensi bakteri dan memberikan dampak negatif bagi organisme perairan. Bakteri perairan banyak dieksplorasi menjadi agen biontrol dan kandidat probiotik potensial untuk meningkatkan kesehatan organisme perairan, efisiensi pakan, dan resistensi penyakit terhadap patogen akuakultur. Namun, isolate lokal belum banyak dieksplorasi dan digunakan untuk meningkatkan produktivitas sektor akuakultur. Oleh karena itu, penelitian ini bertujuan untuk menentukan konsentrasi penghambatan minimum senyawa antibakteri dari ekstrak supernatan terhadap patogen akuakultur dan mengkarakterisasi viabilitas bakteri perairan yang berpotensi diaplikasikan sebagai probiotik dalam pakan. Empat isolat dari beberapa lingkungan perairan di Indonesia (*Pseudomonas* sp. S1.1, *Pseudomonas* sp. S1.2, *Pseudomonas* sp. SL1.1, dan *Bacillus subtilis* KM16) digunakan untuk mengkarakterisasi komponen antibakteri dan menentukan ketahanan bakteri pada pakan. Ekstrak etil asetat dari semua isolat menunjukkan aktivitas antibakteri yang lebih baik terhadap *Aeromonas hydrophila* dan *Vibrio vulnificus* dibandingkan dengan ekstrak kloroform dan diklorometana, dimana ekstrak etil asetat dari *Bacillus subtilis* KM16 menunjukkan aktivitas antibakteri terkuat. Semua isolat *Pseudomonas* sp. lebih efektif melawan *V. vulnificus* (40 mg/mL) dan *Bacillus subtilis* KM16 lebih efektif melawan *A. hydrophila* (20 mg/mL), sebagaimana dibuktikan oleh konsentrasi hambat minimum ekstrak etil asetat dari isolat-isolat tersebut. Pada penelitian ini, *Bacillus subtilis* KM16 memiliki viabilitas yang stabil dalam pakan dibandingkan dengan semua isolat *Pseudomonas* sp.

Kata kunci: Akuakultur, Antibakteri, *Bacillus*, Biokontrol, *Pseudomonas*

**Kontributor Utama*

INTRODUCTION

Aquaculture is one of the significant food-producing sectors in the world. Aquaculture provides great opportunities to accomplish food security and create economic growth. However, with the increasing accentuation and commercialization of aquaculture production, the disease of aquatic organisms is a major problem caused by improper aquaculture husbandry practices and outbreaks of bacterial infections (Akter *et al.,* 2016). Antibiotics are commonly used for disease control of aquatic organisms by kill or inhibit the growth of pathogenic bacteria. Nevertheless, the massive use of antibiotics can lead to the development of antibiotic-resistant bacteria and confer unpleasant impacts to aquatic organisms, such as accretion of those antibiotics in tissues, reduction of beneficial microbiota, and immune suppression (Akter *et al.,* 2016; Chen *et al.,* 2017). Continuous exposure to antibiotics can be toxic to unprotected workers. Furthermore, these antibiotic residues can remain on fish meat and cause health effects on consumers, such as allergies and resistance to human microbiota (Rasul and Majumdar, 2017; Okocha *et al.,* 2018). Due to those threats, the improvement of new, safe, and effective antibacterial agents is required for the health maintenance of aquatic organisms in aquaculture.

Aquatic environments possess a wide range of habitats that exhibit an enormous pool of microbial biodiversity, in which novel natural products can be discovered. Aquatic bacteria are increasingly becoming a potential source in the search for antimicrobial compounds. Antibacterial compounds produced by aquatic bacteria have more unique structures and potent bioactivities which are not found in their terrestrial counterparts due to the complex living circumstance and diversity of species (Jeganathan *et al.,* 2013).

Aquatic bacteria have been discovered as biocontrol agent and potential probiotic candidates, which are a good alternative to replace the use of antibiotics in aquaculture. Probiotics have the ability to improve the health of aquatic organisms, feed efficiency, and disease resistance to aquaculture pathogens by producing antimicrobial compounds (Cruz *et al.,* 2012). According to Prem *et al.,* (2011), potential marine bacteria of genus *Alteromonas* sp., *Streptomyces* sp., *Vibrio* sp., *Bacillus* sp., *Flavobacterium* sp., and *Pseudomonas* sp. showed antimicrobial activity against three major aquaculture pathogens, namely *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Aeromonas hydrophila*. Those bacteria could be applied as probiotics in the aquaculture of fishes, crustaceans, and mollusks against aquaculture pathogens without undesirable side effects. Therefore, the use of aquatic bacteria as biocontrol agent should be seen as an important step in aquaculture sustainability. Besides, probiotic bacteria that is added to the feed must still survive during the packaging, storage, and application in uncontrolled fishery environments, which is characterized by the high number of living microorganisms based on culture-based methods or *in vivo* tests (Pérez-Sánchez *et al*., 2013; Hosain and Liangyi, 2020).

In previous researches by Giovani (2016) and Magdalena *et al.,* (2020), seven isolates from several aquatic environments in Indonesia had been isolated and identified as *Bacillus subtilis* KM16 and *Pseudomonas* spp. (six isolates). These bacteria were isolated from Merah Crater Lake, and waterfall. Both locations have not been explored for microbial potency to be developed as probiotics. Isolated *Bacillus subtilis* KM16 and *Pseudomonas* spp show biocontrol activity, both antagonistic activity via extracellular substances and probiotic activity. Both bacteria show antagonistic activity against some pathogenic Gram-positive and Gram-negative bacteria, mold, and yeast. Furthermore, the bacterial isolates also exhibit antagonistic activity against aquaculture pathogens (*A. hydrophila* and *V. vulnificus*) in different culture conditions (Shirley 2017). However, the possibility to be applied as a part of probiotics feed is not studied yet. This research aimed to determine minimum inhibitory concentrations of their antibacterial compounds against aquaculture pathogens and characterize aquatic bacteria by their viability in the feed.

MATERIAL AND METHODS

Preparation of bacterial isolates

Four isolates had been isolated from several aquatic environments in Indonesia (Giovani 2016; Magdalena *et al.*, 2020). Isolates were cultivated on Nutrient Agar (NA) and incubated at their optimum condition (Table 1). We were also used *Aeromonas hydrophila* ATCC 7966 and *Vibrio vulnificus* ATCC 27562 as pathogenic bacteria. *A. hydrophila* were cultivated on NA at 28°C for 24 hours. *V. vulnificus* were streaked onto Brain Heart Infusion Agar (BHIA) and incubated at 28°C for 24 hours.

Table 1. Optimum growth condition of the aquatic bacterial isolates *(Kondisi optimum pertumbuhan isolat bakteri akuatik)*

Isolate *(Isolat)*	**Origin** *(Asal)*	**Medium** *(Media)*	**Temperature** *(Suhu)*	**Incubation time** *(Waktu inkubasi)*
Bacillus subtilis KM16	Merah Crater Lake, Bogor *(Kawah Merah, Bogor)*	NA	37°C	24 hours *(24 jam)*
Pseudomonas sp. S1.1	Darmaloka Waterfall, West Java *(Air Terjun Darmaloka, Jawa Barat)*	NA	28°C	24 hours *(24 jam)*
Pseudomonas sp. S1.2	Darmaloka Waterfall, West Java *(Air Terjun Darmaloka, Jawa Barat)*	NA	28°C	24 hours *(24 jam)*
Pseudomonas sp. SL1.1	Sawer Rahmat Waterfall, West Java *(Air Terjun Sawer Rahmat, Jawa Barat)*	NA	28°C	24 hours *(24 jam)*

Production of the aquatic bacteria culture broth

This method was adopted from Abdul-Hussein and Atia (2016). Each isolate was grown in NB and measured to reach 0.5 McFarland (OD_{600} = 0.132). For extracellular production, a total of 20 mL of each standardized isolate suspension was inoculated into 100 mL of NB and incubated at 28°C for 48 hours with 120 rpm agitation. The production was scaled up to 1200 mL for each isolate. Each suspension was centrifuged at 7513 × g for 20 minutes. Each supernatant was collected and tested against pathogens by using well diffusion method.

Extraction of the aquatic bacteria culture broth

This method was adopted from Hayashida-Soiza *et al.* (2008) with modifications in various organic solvent. Extraction was done by using the liquid-liquid extraction method. As mentioned above, 1200 mL of each supernatant was mixed with chloroform, dichloromethane, and ethyl acetate with a volume ratio of 1:1 (v/v). Each mixture was stirred in a separating funnel for 5 minutes and allowed to sit still to separate organic and aqueous phases. Each organic phase was collected and separated from the solvents using a rotary evaporator at 40°C with different pressure based on the solvents. Each crude extract was dried in a fume hood for 24 hours and diluted with 1% (v/v) DMSO. The percentage of each crude extract yield was determined. Yield percentage = extract's weight / volume of supernatant x 100%.

Anbacterial activity assay

This method was adopted from Balouiri *et al.* (2016). Antibacterial activity assay was done by using disc diffusion method. *A. hydrophila* and *V. vulnificus* were grew at their optimum condition and measured to reach 0.5 McFarland (OD_{600} = 0.132). A total of 100 µL of each standardized pathogen suspension was spotted on Mueller Hin-

ton Agar and streaked it three times continuously. Blank discs were placed on the agar surface. A total of 10 µL of each extract from the previous step was loaded into each disc. Positive controls were prepared by using aztreonam (30 µg) (Oxoid Ltd., Hampshire, UK) and nalidixic acid (30 µg) (Oxoid Ltd., Hampshire, UK). Negative control was prepared by using 1% (v/v) DMSO. Agar plates were incubated at 28°C for 24 hours. Positive results were determined by measuring the size of clear zones around the discs. This step was done in triplicate.

Minimum inhibitory concentration (MIC) assay

This method was adopted from Hayashida-Soiza *et al.* (2008) with modifications in various crude extract concentrations. MIC assay was done by using disc diffusion method. Two-fold serial dilution of each extract was done to produce extract with several concentrations from 640 mg/mL to 0.0012 mg/mL. *A. hydrophila* and *V. vulnificus* were grew at their optimum condition and measured to reach 0.5 McFarland (OD_{600} = 0.132). A total of 100 µL of each standardized pathogen suspension was spotted on MHA and streaked it three times continuously. Blank discs were placed on the agar surface. A total of 10 µL of each extract with different concentrations was loaded into each disc. Negative controls were prepared by using NB, BHI, and 1% (v/v) DMSO. Agar plates were incubated at 28°C for 24 hours. MICs were determined as the lowest concentration of extracts to inhibit the growth of pathogens (smallest clear zones). This step was done in triplicate.

Determination of the isolate viability in feed

This method was adopted from Aly *et al.* (2008) with modifications. Each isolate was grown in Nutrient Broth (NB) and measured to reach 0.5 McFarland (OD_{600} = 0.132). A total of 10 mL of each standardized isolate suspension was centrifuged at 12396 × g for 10 minutes. Each pellet was collected and resuspended with 10 mL of sterile physiological saline solution (0.85% w/v NaCl). A total of 4 mL of each suspension was added to 10 g of commercial fish feed (30% of crude protein, 3% of crude fat, 4% of crude fiber, and 12% of crude ash) and dried in oven at 40°C for 24 hours. One half of each feed was stored at 4°C, while the other half was stored at 25°C. The viability of isolates in feed were assessed every week for 5 weeks of storage. A total of 1 g of each feed was homogenized in 9 mL of 0.85% (w/v) NaCl. Ten-fold serial dilution of each suspension was carried out and 100 µL of each diluted suspension was spread onto NA and incubated at 28°C for 24 hours. Negative control was prepared by using feed without the addition of isolates. Colony counts were done to estimate the number of viable bacteria. Experiments were performed in triplicates.

RESULTS

Antibacterial activity

Supernatant from all isolates were extracted with chloroform, dichloromethane, and ethyl acetate. Supernatant extract yield percentages from all isolates were varied (Table 2). All extracts with similar concentration (± 600 mg/mL) were tested against *A. hydrophila* and *V. vulnificus*. Ethyl acetate extracts from all isolates showed better antibacterial activity than chloroform and dichloromethane extracts, as well cell-free supernatants (Table 3). None of chloroform and dichloromethane extracts from all isolates of *Pseudomonas* sp. showed antibacterial activity against *A. hydrophila*. Ethyl acetate extracts from *Pseudomonas* sp. SL1.1 and *Bacillus subtilis* KM16 showed greater inhibition zones against *A. hydrophila* and *V. vulnificus* compared to cell-free supernatant (Figure 1). There were new activities after extraction shown by ethyl acetate extracts from all isolates of *Pseudomonas* sp. against *A. hydrophila* and all extracts from *Bacillus subtilis* KM16 against *V. vulnificus*. Overall, all extracts from all isolates showed greater antimicrobial activity against *V. vulnificus* than *A. hydrophila*.

Table 2. The percentages extraction yield of aquatic bacterial isolates *(Persentase hasil ekstraksi isolat bakteri akuatik)*

Isolates *(Isolat)*	**Yield (% w/v)** *(Hasil (% b/v))*		
	Chloroform *(Kloroform)*	**Dichloromethane** *(Diklorometana)*	**Ethyl acetate** *(Etil asetat)*
Pseudomonas sp. S1.1	4.06	3.69	5.10
Pseudomonas sp. S1.2	3.68	6.76	7.95
Pseudomonas sp. SL1.1	3.57	6.63	6.58
Bacillus subtilis KM16	3.14	19.98	7.60

Table 3. Antibacterial activities of several extracts of aquatic bacterial isolates *(Aktivitas antibakteri dari beberapa ekstrak isolat bakteri akuatik)*

Pathogens *(Patogen)*	**Isolates** *(Isolat)*	**Zone of inhibition (mm)** *(Zona hambat (mm))*			
		S	**C**	**D**	**E**
A. hydrophila	*Pseudomonas* sp. S1.1	-	-	-	2
	Pseudomonas sp. S1.2	-	-	-	2
	Pseudomonas sp. SL1.1	-	-	-	3
	Bacillus subtilis KM16	7	7	9	13
V. vulnificus	*Pseudomonas* sp. S1.1	5	6*	3	4
	Pseudomonas sp. S1.2	4	-	1	4
	Pseudomonas sp. SL1.1	5	1	1	7
	Bacillus subtilis KM16	-	3*	5*	6

S = cell-free supernatant, C = chloroform extract, D = dichloromethane extract, E = ethyl acetate extract.
*Zone of inhibition was not clear
*S = supernatan bebas sel, C = ekstrak kloroform, D= ekstrak diklorometana, E= ekstrak etil asetat. * Zona hambat tidak jernih*

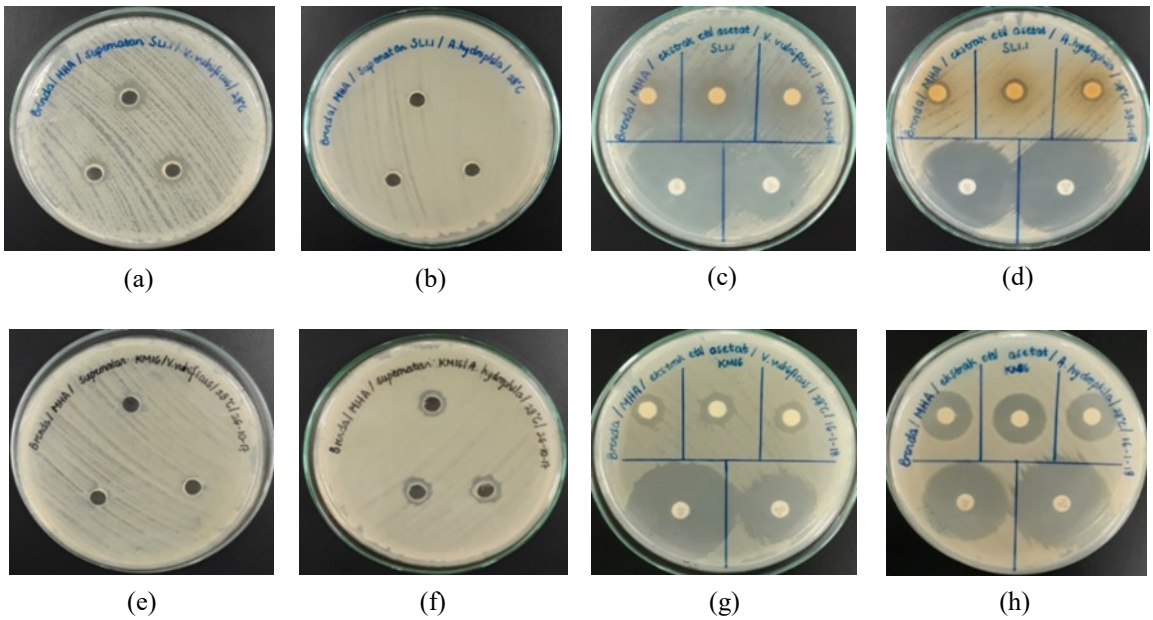

Figure 1. Antibacterial activity of *Pseudomonas* sp. SL1.1 (a-d) and *Bacillus subtilis* KM16 (e-h) against aquaculture pathogens. (a,e) cell-free supernatant against *V. vulnificus*; (b,f) cell-free supernatant against *A. hydrophila*; (c,g) ethyl acetate extract against *V. vulnificus*; (d,h) ethyl acetate extract against *A. hydrophila*. NA= nalidixic acid (30μg). ATM = aztreonam (30μg).
Aktivitas antibakteri Pseudomonas sp. *SL1.1 (a-d) dan Bacillus subtilis KM16 (e-h) terhadap patogen akuakultur. (a,e) supernatan bebas sel terhadap V. vulnificus; (b,f) supernatan bebas sel terhadap A. hydrophila; (c,g) ekstrak etil asetat terhadap V. vulnificus; (d,h) ekstrak etil asetat terhadap A. hydrophila. NA= asam nalidiksat (30µg). ATM = aztreonam (30µg).*

Minimum inhibitory concentration (MIC)

Ethyl acetate extracts from *Pseudomonas* sp. S1.1 and SL1.1, and *Bacillus subtilis* KM16 were used to determine their MIC against *A. hydrophila* and *V. vulnificus*. Ethyl acetate extracts from *Pseudomonas* sp. S1.1 and SL1.1 showed similar MIC against both pathogens, in which 160 mg/mL was the MIC for *A. hydrophila* and 40 mg/mL was the MIC for *V. vulnificus*. Ethyl acetate extract from *Bacillus subtilis* KM16 could inhibit the growth of *A. hydrophila* and *V. vulnificus* with the MIC of 20 mg/mL and 160 mg/mL respectively (Table 4).

Table 4. Minimum inhibitory concentration (MIC) of ethyl acetate extracts of aquatic bacterial against aquaculture pathogens *(Konsentrasi hambat minimum (KHM) dari ekstrak etil asetat bakteri akuatik terhadap patogen akuakultur)*

Ethyl acetate extracts ***(Ekstrak etil asetat)***	**Pathogens** ***(Patogen)***	**MIC (mg/mL)** ***(KHM (mg/mL))***
Pseudomonas sp. S1.1	*A. hydrophila*	160
	V. vulnificus	40
Pseudomonas sp. SL1.1	*A. hydrophila*	160
	V. vulnificus	40
Bacillus subtilis KM16	*A. hydrophila*	20
	V. vulnificus	160

Viability of the isolates in feed

All isolates were added into commercial fish feed, dried at 40°C for 24 hours, and stored for 5 weeks. None of *Pseudomonas* sp. isolates survived at both 4°C and 25°C since the first week of storage. Meanwhile, *Bacillus subtilis* KM16 survived at both 4°C and 25°C over the five weeks of storage (Figure 2).

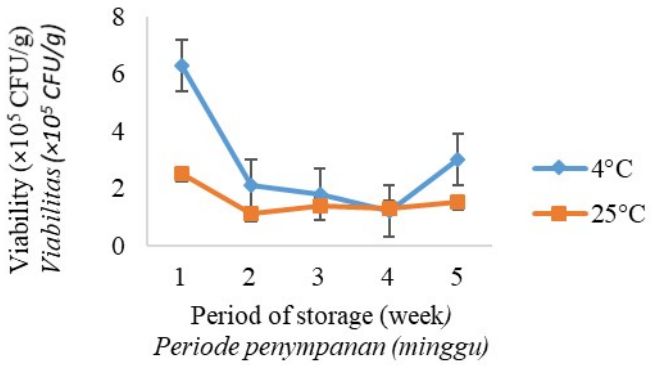

Figure 2. The viability of *Bacillus subtilis* KM16 in feed. n=3; error bar was presented as Standard Deviation *(Viabilitas Bacillus subtilis KM16 pada pakan. n=3; bar kesalahan menunjukkan Standar Deviasi)*

DISCUSSION

Pseudomonas sp. and *Bacillus* sp. are used for various biotechnological applications, including biocontrol agents and probiotic supplementation in animal feed for aquaculture. Screening of extracellular antibacterial activity of all isolates against aquaculture pathogens was undertaken in previous research by Shirley (2017). Cell-free supernatant of *Pseudomonas* sp. S1.1, S1.2, and SL1.1 showed antibacterial activity against *V. vulnificus*. Meanwhile, cell-free supernatant of *Bacillus subtilis* KM16 showed antibacterial activity against *A. hydrophila*. All isolates also had amylase and protease activity, which are potential to be applied as biocontrol agents and probiotics to improve digestive enzyme activity of aquatic organisms.

Ethyl acetate, dichloromethane, and chloroform were used to extract antibacterial compounds from all isolates, in which each isolate showed various percentages of supernatant extract yield (Table 2). Ethyl acetate is more polar than chloroform and chloroform is more polar than dichloromethane (Houghton and Raman 2012). Overall, ethyl acetate extracts had better yield percentages than other solvents. Ethyl acetate could increase the solubility of secondary metabolites and exhibited more antimicrobial activity (Chairman *et al.*, 2012). However, dichloromethane extract from *Bacillus subtilis* KM16 showed the highest yield percentage. This could be related to the ability of *Bacillus* sp. to produce non polar antibacterial compounds such as fatty acids (Mondol *et al.*, 2013).

In this research, solvent with higher polarity was the most suitable for extraction, but the best solvents can not be specified or generalized for all of the antibacterial compounds. Antibacterial compounds can be extracted with different solvents based on the polarity of solvents and the suitability of antibacterial compounds with the solvents (Veronica *et al.*, 2014).

Ethyl acetate extracts from all isolates had better antibacterial activity against *A. hydrophila* and *V. vulnificus* (Table 3); indicating those extracted antibacterial compounds were polar. This result was related with previous study reported by Mohan *et al.* (2016), in which ethyl acetate extract from marine sponge-associated *Bacillus* sp. showed significant antibacterial activity against *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, *A. salmonicida*, *Proteus mirabilis*, *Citrobacter brackii*, *Flavobacterium* sp., and *Edwardsiella* sp; compared with n-hexane, chloroform, dichloromethane, methanol, and DMSO extracts. Prem *et al.* (2011) also found that ethyl acetate extract from

marine bacteria (*Streptomyces* sp., *Vibrio* sp., *Bacillus* sp., *Flavobacterium* sp., *and Pseudomonas* sp.) showed antibacterial activity against *A. hydrophila*, *V. harveyi*, and *V. parahaemolyticus*. Some isolates showed new and better antibacterial activity after extraction. Extraction will increase the purity and activity of antibacterial compounds, so that inhibition zones after extraction will be greater than before extraction (Veronica *et al.*, 2014).

All isolates were able to produce antibacterial compounds which could be extracted by ethyl acetate and had antibacterial activity against *A. hydrophila* and *V. vulnificus*. Ethyl acetate extracts from *Pseudomonas* sp. S1.1 and SL1.1 were more effective against *V. vulnificus* with the MIC of 40 mg/mL. Meanwhile, ethyl acetate extract from *Bacillus subtilis* KM16 was more effective against *A. hydrophila* with the MIC of 20 mg/mL (Table 4). The effectiveness of antibacterial compounds depends on the sensitivity of bacterial strains towards specific types of antibacterial compounds and the mechanisms of their activities including the inhibition of gene expression and cellular biomolecules synthesis (Isa *et al.*, 2017).

Bacillus sp. and *Pseudomonas* sp. produce various antimicrobial compounds that had shown antagonistic activity against many pathogenic microorganisms. Both bacteria are frequently used as biocontrol agents and probiotics for the prevention or treatment of animal infections. *Bacillus* sp. produce versatile secondary metabolites including bacteriocins, lipopeptides, polypeptides, polyketides, lipoamides, macrolactones, isocoumarins, and fatty acids (Mondol *et al.*, 2013). Pyrrolo[1,2-*a*]pyrazine -1,4-dione, hexahydro- from marine sponge-associated *Bacillus* sp. extracted with ethyl acetate had antibacterial acitivity against *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, and *A. salmonicida* (Mohan *et al.*, 2016). O-heterocyclic pyrans from marine seaweed-associated *B. subtilis* extracted with ethyl acetate had antibacterial activity against *A. hydrophila*, *V. vulnificus*, and *V. parahaemolyticus* (Chakraborty *et al.*, 2017). Aminocoumacin A from marine *B. pumilus* extracted with methanol had antibacterial activity against *V. vulnificus* by disrupting the cell membrane (Gao *et al.*, 2017). Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- from marine *B. pumilus* extracted with ethyl acetate had antibacterial activity against *A. hydrophila* by disrupting the cell wall, granulating in the bacterial cell, and destructing the nucleus (Malash *et al.*, 2016).

Pseudomonas sp. produces diverse bioactive substances including pyocyanin, pyoverdine, pyrrole, phenazine, phenanthren, phthalate, benzaldehyde, quinoline, and quinolone (Isnansetyo and Kamei, 2009). Cell-free supernatant and crude ethyl acetate extract from marine *P. stutzeri* had antibacterial activity against *V. harveyi*, *V. cholerae*, *V. alginolyticus*, *V. damseal*, and *V. fluvialis* (Uzair *et al.*, 2006). A bacteriocin and a siderophore from marine *P. cepacia* had antibacterial activity against *A. hydrophila*, *A. salmonicida*, *V. vulnificus*, and *V. anguillarum* (Bourouni *et al.*, 2010). Protein fractions from extracelullar products of *Pseudomonas* sp. could repress the growth of *A. hydrophila* and *V. harveyi*, related to the content of antibiotics, bacteriocins, siderophores, lysozyme, and protease (Hardi *et al.*, 2016). Ethyl acetate extract from marine sponge-associated *Pseudomonas* sp. showed the presence of alkaloids, quinones, flavonoids, and flavonyl glicosides (further identified as chromophoric substances) that had antibacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) (Skariyachan *et al.*, 2013). Besides, naphth [2,3-B] azet-2 (1H) -one, 1-phenyl- from soil *Pseudomonas* sp. extracted with methanol had antibacterial activity against human pathogenic bacteria including *P. aeruginosa*, *B. subtilis*, *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli* (Verma *et al.*, 2015).

In addition to secondary metabolites, the inhibitory mechanism of *Bacillus* sp. as a biocontrol includes inhibiting the quorum sensing process in pathogenic bacteria, competing for nutrients, and producing organic acid molecules (Kuebutornye *et al*., 2020). In *Pseudomonas* M162, the inhibitory activity of the bacterium *Flavobacterium psychrophilum* as a pathogen in rainbow trout (*Oncorhynchus mykiss*) is due to its ability to colonize the digestive tract and to stimulate leukocyte and immunoglobulin activity in the immune system. (Korkea-aho *et al.*, 2012).

In this research, all isolates were added to the

feed. It is expected that those isolates survive when the feed is given to aquatic organisms so that they can be applied as probiotics for disease control in the aquaculture environment. From these results, *Bacillus subtilis* KM16 was more withstand to drying and storage conditions of feed than all isolates of *Pseudomonas* sp. These results were related to another study by Aly *et al*. (2008), in which *B. pumilus* survived in feed after dried at 45°C and stored at 4°C and 25°C for 5 weeks of storage. Drying could eliminate water content in the feed, thus increasing the stability of isolates over a long period of time, but none of *Pseudomonas* sp. isolates survived after drying. *Bacillus subtilis* KM16 showed its viability at both 4°C and 25°C over the five weeks of storage, but those isolates showed higher CFU/g in the feed stored at 4°C (Figure 2). Storage of the feed at 4°C could prevent the loss of probiotics viability (Campbell *et al.*, 2017).

The low ability of *Pseudomonas* to survive on feed can occur because of the heating process at 40°C for 24 hours. Therefore, further research is needed with the use of encapsulation in making functional feed. Encapsulation in the application of probiotics in an aquaculture environment plays a role in increasing the availability of living bioactive in the desired amount, helping to deliver it to specific targets from the body, and increasing resistance through extreme conditions, such as low pH in the gastrointestinal tract of some fish (Dezfooli *et al*., 2018). *Lactobacillus rhamnosus* GG encapsulated with alginate matrix, or a combination of skim-alginate milk could survive the storage process at room temperature for 14 days and successfully passed simulated gastric conditions at pH 1.5 for 3 hours and bile salts. *L. rhamnosus* GG survival ability without encapsulation was significantly reduced in all of these treatments (Pirarat *et al*., 2015).

Spore-forming *Bacillus* sp. has advantages to be used as probiotics compared with other non spore-forming bacteria. Spore formation, growth conditions, and phenotypic traits can affect the viability of *Bacillus* sp. According to FAO (2016), spores of *Bacillus* sp. are resistant to physical and environmental factors, so that the bacteria can maintain their viability during feed handling and storage. Various spesies of *Bacillus* are increasingly popular to be used as probiotics in animal feed. *B. subtilis, B. licheniformis, B. coagulans, B. polymyxa,* and *B. megaterium* were identified as safe due to the absence of enterotoxins and emetic toxins.

Bacillus sp. supplementation in aquaculture could improve growth performance, immune response, digestive enzymes activity, and disease resistance of aquatic organisms. *Bacillus* sp. are more stable under storage conditions and industrial processes which make them more suitable for feed supplementation (Buruiana *et al.*, 2014). Commercial probiotic *Bacillus* sp. (*B. subtilis*, *B. licheniformis*, *B. polymyxa*, *B. laterosporus*, and *B. circulans*) improved digestive enzyme activity, survival, and growth of white shrimp (Ziaei-Nejad *et al.*, 2006). Spores of *B. subtilis* strains reduced catfish mortality significantly (Ran *et al.*, 2012). *Bacillus* sp. spores (*B. subtilis*, *B. clausii*, *B. cereus*, *B. coagulans*, and *B. licheniformis*) could be stored at room temperature in desiccated form without any impact on feasibility and survived at low gastric pH of aquatic organisms (Cutting, 2011).

From this results, *Bacillus subtilis* KM16 had better antibacterial activity against *A. hydrophila* and *V. vulnificus* than all isolates of *Pseudomonas* sp. *Bacillus* sp. are good prospect as biocontrol agents for aquaculture because they are plentiful in a wide range of environments and habitats, they produce antibacterial compounds with well-documented antagonistic activity against pathogenic microorganisms, and they form spores that can be easily composed in animal feed and have high viability than vegetative cells (Mondol *et al.*, 2013). Further study on the purification and characterization of antibacterial compounds from all isolates is needed to understand their types, structures, and action mechanisms.

CONCLUSION

Ethyl acetate extracts from all isolates showed better antibacterial activity than chloroform and dichloromethane extracts, in which ethyl acetate extract from *Bacillus subtilis* KM16 showed the strongest antibacterial activity against *A. hydrophila* and *V. vulnificus.* Based on the MIC results of

ethyl acetate extracts, *Pseudomonas* sp. isolates were more effective against *V. vulnificus* (40 mg/mL) and *Bacillus subtilis* KM16 was more effective against *A. hydrophila* (20 mg/mL). *Bacillus subtilis* KM16 exhibited stable viability in feed than all isolates of *Pseudomonas* sp.

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Pedoman Penulisan Naskah Berita Biologi

Berita Biologi adalah jurnal yang menerbitkan artikel kemajuan penelitian di bidang biologi dan ilmu-ilmu terkait di Indonesia. Berita Biologi memuat karya tulis ilmiah asli berupa makalah hasil penelitian, komunikasi pendek dan tinjauan kembali yang belum pernah diterbitkan atau tidak sedang dikirim ke media lain. Masalah yang diliput harus menampilkan aspek atau informasi baru.

Tipe naskah

1. **Makalah lengkap hasil penelitian (*original paper*)**
   Naskah merupakan hasil penelitian sendiri yang mengangkat topik yang *up to date*. Tidak lebih dari 15 halaman termasuk tabel dan gambar. Pencantuman lampiran seperlunya, namun redaksi berhak mengurangi atau meniadakan lampiran.
2. **Komunikasi pendek (*short communication*)**
   Komuniasi pendek merupakan makalah hasil penelitian yang ingin dipublikasikan secara cepat karena hasil termuan yang menarik, spesifik dan atau baru, agar dapat segera diketahui oleh umum. Hasil dan pembahasan dapat digabung.
3. **Tinjauan kembali (*review*)**
   Tinjauan kembali merupakan rangkuman tinjauan ilmiah yang sistematis-kritis secara ringkas namun mendalam terhadap topik penelitian tertentu. Hal yang ditinjau meliputi segala sesuatu yang relevan terhadap topik tinjauan yang memberikan gambaran '*state of the art*', meliputi temuan awal, kemajuan hingga issue terkini, termasuk perdebatan dan kesenjangan yang ada dalam topik yang dibahas. Tinjauan ulang ini harus merangkum minimal 30 artikel.

Struktur naskah

1. **Bahasa**
   Bahasa yang digunakan adalah Bahasa Indonesia atau Inggris yang baik dan benar.
2. **Judul**
   Judul diberikan dalam bahasa Indonesia dan inggris. Judul ditulis dalam huruf tegak kecuali untuk nama ilmiah yang menggunakan bahasa latin, Judul harus singkat, jelas dan mencerminkan isi naskah dengan diikuti oleh nama serta alamat surat menyurat penulis dan alamat email. Nama penulis untuk korespondensi diberi tanda amplop cetak atas (*superscript*). Jika penulis lebih dari satu orang bagi pejabat fungsional penelitian, pengembangan agar menentukan status sebagai kontributor utama melalui penandaan simbol dan keterangan sebagai kontributor utama dicatatan kaki di halaman pertama artikel.
3. **Abstrak**
   Abstrak dibuat dalam dua bahasa, bahasa Indonesia dan Inggris. Abstrak memuat secara singkat tentang latar belakang, tujuan, metode, hasil yang signifikan, kesimpulan dan implikasi hasil penelitian. Abstrak berisi maksimum 200 kata, spasi tunggal. Di bawah abstrak dicantumkan kata kunci yang terdiri atas maksimum enam kata, dimana kata pertama adalah yang terpenting. Abstrak dalam Bahasa Inggris merupakan terjemahan dari Bahasa Indonesia. Editor berhak untuk mengedit abstrak demi alasan kejelasan isi abstrak.
4. **Pendahuluan**
   Pendahuluan berisi latar belakang, permasalahan dan tujuan penelitian. Perlu disebutkan juga studi terdahulu yang pernah dilakukan terkait dengan penelitian yang dilakukan.
5. **Bahan dan cara kerja**
   Bahan dan cara kerja berisi informasi mengenai metode yang digunakan dalam penelitian. Pada bagian ini boleh dibuat sub-judul yang sesuai dengan tahapan penelitian. Metoda harus dipaparkan dengan jelas sesuai dengan standar topik penelitian dan dapat diulang oleh peneliti lain. Apabila metoda yang digunakan adalah metoda yang sudah baku cukup ditulis sitasinya dan apabila ada modifikasi maka harus dituliskan dengan jelas bagian mana dan hal apa yang dimodifikasi.
6. **Hasil**
   Hasil memuat data ataupun informasi utama yang diperoleh berdasarkan metoda yang digunakan. Apabila ingin mengacu pada suatu tabel/ grafik/diagram atau gambar, maka hasil yang terdapat pada bagian tersebut dapat diuraikan dengan jelas dengan tidak menggunakan kalimat 'Lihat Tabel 1'. Apabila menggunakan nilai rata- rata maka harus menyertakan pula standar deviasinya.
7. **Pembahasan**
   Pembahasan bukan merupakan pengulangan dari hasil. Pembahasan mengungkap alasan didapatkannya hasil dan arti atau makna dari hasil yang didapat tersebut. Bila memungkinkan, hasil penelitian ini dapat dibandingkan dengan studi terdahulu.
8. **Kesimpulan**
   Kesimpulan berisi infomasi yang menyimpulkan hasil penelitian, sesuai dengan tujuan penelitian, implikasi dari hasil penelitian dan penelitian berikutnya yang bisa dilakukan.
9. **Ucapan terima kasih**
   Bagian ini berisi ucapan terima kasih kepada suatu instansi jika penelitian ini didanai atau didukungan oleh instansi tersebut, ataupun kepada pihak yang membantu langsung penelitian atau penulisan artikel ini.
10. **Daftar pustaka**
    Tidak diperkenankan untuk mensitasi artikel yang tidak melalui proses *peer review*. Apabila harus menyitir dari "laporan" atau "komunikasi personal" dituliskan '*unpublished*' dan tidak perlu ditampilkan di daftar pustaka. Daftar pustaka harus berisi informasi yang *up to date* yang sebagian besar berasal dari *original papers* dan penulisan terbitan berkala ilmiah (nama jurnal) tidak disingkat.

Format naskah

1. Naskah diketik dengan menggunakan program Microsoft Word, huruf New Times Roman ukuran 12, spasi ganda kecuali Abstrak spasi tunggal. Batas kiri-kanan atas-bawah masing-masing 2,5 cm. Maksimum isi naskah 15 halaman termasuk ilustrasi dan tabel.
2. Penulisan bilangan pecahan dengan koma mengikuti bahasa yang ditulis menggunakan dua angka desimal di belakang koma. Apabila menggunakan Bahasa Indonesia, angka desimal ditulis dengan menggunakan koma (,) dan ditulis dengan menggunakan titik (.) bila menggunakan bahasa Inggris. Contoh: Panjang buku adalah 2,5 cm. Lenght of the book is 2.5 cm. Penulisan angka 1-9 ditulis dalam kata kecuali bila bilangan satuan ukur, sedangkan angka 10 dan seterusnya ditulis dengan angka. Contoh lima orang siswa, panjang buku 5 cm.
3. Penulisan satuan mengikuti aturan *international system of units*.
4. Nama takson dan kategori taksonomi ditulis dengan merujuk kepada aturan standar yang diakui. Untuk tumbuhan menggunakan *International Code of Botanical Nomenclature* (ICBN), untuk hewan menggunakan International *Code of Zoological Nomenclature* (ICZN), untuk jamur *International Code of Nomenclature for Algae*, *Fungi and Plant* (ICFAFP), *International Code of Nomenclature of Bacteria* (ICNB), dan untuk organisme yang lain merujuk pada kesepakatan Internasional. Penulisan nama takson lengkap dengan nama author hanya dilakukan pada bagian deskripsi takson, misalnya pada naskah taksonomi. Penulisan nama takson untuk bidang lainnya tidak perlu menggunakan nama author.
5. Tata nama di bidang genetika dan kimia merujuk kepada aturan baku terbaru yang berlaku.

6. Untuk range angka menggunakan en dash (–), contohnya pp.1565–1569, jumlah anakan berkisar 7–8 ekor. Untuk penggabungan kata menggunakan hyphen (-), contohnya: masing-masing.
7. Ilustrasi dapat berupa foto (hitam putih atau berwarna) atau gambar tangan (*line drawing*).
8. Tabel
   Tabel diberi judul yang singkat dan jelas, spasi tunggal dalam bahasa Indonesia dan Inggris, sehingga Tabel dapat berdiri sendiri. Tabel diberi nomor urut sesuai dengan keterangan dalam teks. Keterangan Tabel diletakkan di bawah Tabel. Tabel tidak dibuat tertutup dengan garis vertikal, hanya menggunakan garis horisontal yang memisahkan judul dan batas bawah.
9. Gambar
   Gambar bisa berupa foto, grafik, diagram dan peta. Judul gambar ditulis secara singkat dan jelas, spasi tunggal. Keterangan yang menyertai gambar harus dapat berdiri sendiri, ditulis dalam bahasa Indonesia dan Inggris. Gambar dikirim dalam bentuk .jpeg dengan resolusi minimal 300 dpi, untuk *line drawing* minimal 600dpi.
10. Daftar Pustaka
    Sitasi dalam naskah adalah nama penulis dan tahun. Bila penulis lebih dari satu menggunakan kata 'dan' atau *et al*. Contoh: (Kramer, 1983), (Hamzah dan Yusuf, 1995), (Premachandra *et al*., 1992). Bila naskah ditulis dalam bahasa Inggris yang menggunakan sitasi 2 orang penulis maka digunakan kata 'and'. Contoh: (Hamzah and Yusuf, 1995). Jika sitasi beruntun maka dimulai dari tahun yang paling tua, jika tahun sama maka dari nama penulis sesuai urutan abjad. Contoh: (Anderson, 2000; Agusta *et al*., 2005; Danar, 2005). Penulisan daftar pustaka, sebagai berikut:

    a. **Jurnal**
       Nama jurnal ditulis lengkap.
       Agusta, A., Maehara, S., Ohashi, K., Simanjuntak, P. and Shibuya, H., 2005. Stereoselective oxidation at C-4 of flavans by the endophytic fungus *Diaporthe* sp. isolated from a tea plant. *Chemical and Pharmaceutical Bulletin*, *53*(12), pp.1565–1569.
    b. **Buku**
       Anderson, R.C. 2000. *Nematode Parasites of Vertebrates, Their Development and Tramsmission*. 2nd ed. CABI Publishing. New York. pp. 650.
    c. **Prosiding atau hasil Simposium/Seminar/Lokakarya.**
       Kurata, H., El-Samad, H., Yi, T.M., Khammash, M. and Doyle, J., 2001. Feedback Regulation of the Heat Shock Response in *Eschericia coli*. *Proceedings of the 40th IEEE Conference on Decision and Control*. Orlando, USA. pp. 837–842.
    d. **Makalah sebagai bagian dari buku**
       Sausan, D., 2014. Keanekaragaman Jamur di Hutan Kabungolor, Tau Lumbis Kabupaten Nunukan, Kalimanan Utara. Dalam: Irham, M. & Dewi, K. eds. *Keanekaraman Hayati di Beranda Negeri*. pp. 47–58. PT. Eaststar Adhi Citra. Jakarta.
    e. **Thesis, skripsi dan disertasi**
       Sundari, S., 2012. Soil Respiration and Dissolved Organic Carbon Efflux in Tropical Peatlands. *Dissertation*. Graduate School of Agriculture. Hokkaido University. Sapporo. Japan.
    f. **Artikel online.**
       Artikel yang diunduh secara online ditulis dengan mengikuti format yang berlaku untuk jurnal, buku ataupun thesis dengan dilengkapi alamat situs dan waktu mengunduh. Tidak diperkenankan untuk mensitasi artikel yang tidak melalui proses peer review misalnya laporan perjalanan maupun artikel dari laman web yang tidak bisa dipertangung jawabkan kebenarannya seperti wikipedia.
       Himman, L.M., 2002. A Moral Change: Business Ethics After Enron. San Diego University Publication. *http:ethics.sandiego.edu/LMH/oped/Enron/index.asp*. (accessed 27 Januari 2008) bila naskah ditulis dalam bahasa inggris atau (diakses 27 Januari 2008) bila naskah ditulis dalam bahasa indonesia

Formulir persetujuan hak alih terbit dan keaslian naskah
Setiap penulis yang mengajukan naskahnya ke redaksi Berita Biologi akan diminta untuk menandatangani lembar persetujuan yang berisi hak alih terbit naskah termasuk hak untuk memperbanyak artikel dalam berbagai bentuk kepada penerbit Berita Biologi. Sedangkan penulis tetap berhak untuk menyebarkan edisi cetak dan elektronik untuk kepentingan penelitian dan pendidikan. Formulir itu juga berisi pernyataan keaslian naskah yang menyebutkan bahwa naskah adalah hasil penelitian asli, belum pernah dan tidak sedang diterbitkan di tempat lain serta bebas dari konflik kepentingan.

Penelitian yang melibatkan hewan dan manusia
Setiap naskah yang penelitiannya melibatkan hewan (terutama mamalia) dan manusia sebagai obyek percobaan/penelitian, wajib menyertakan *'ethical clearance approval*' yang dikeluarkan oleh badan atau pihak berwenang.

Lembar ilustrasi sampul
Gambar ilustrasi yang terdapat di sampul jurnal Berita Biologi berasal dari salah satu naskah yang dipublikasi pada edisi tersebut. Oleh karena itu, setiap naskah yang ada ilustrasinya diharapkan dapat mengirimkan ilustrasi atau foto dengan kualitas gambar yang baik dengan disertai keterangan singkat ilustrasi atau foto dan nama pembuat ilustrasi atau pembuat foto.

Proofs
Naskah *proofs* akan dikirim ke penulis dan penulis diwajibkan untuk membaca dan memeriksa kembali isi naskah dengan teliti. Naskah proofs harus dikirim kembali ke redaksi dalam waktu tiga hari kerja.

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Naskah dikirim secara online ke website berita biologi: *http://e-journal.biologi.lipi.go.id/index.php/berita_biologi*

Alamat kontak
Redaksi Jurnal Berita Biologi, Pusat Penelitian Biologi-LIPI
Cibinong Science Centre, Jl. Raya Bogor Km. 46 Cibinong 16911
Telp: +61-21-8765067, Fax: +62-21-87907612, 8765063, 8765066,
Email: berita.biologi@mail.lipi.go.id
jurnalberitabiologi@yahoo.co.id atau
jurnalberitabiologi@gmail.com

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SHORT COMMUNICATION