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Eukaryote microbes potential for bioflocs in the swamp aquaculture

Potensi mikrob eukariot untuk bioflok akuakultur lahan rawa payau

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ABSTRACT

Eucaryote microbes have an important role in forming bioflocs in brackishwater aquaculture ponds. Bioflocs become potential live feed for milkfish or crustacea. This study aimed to identify the potential of eukaryotic microbes in the brackishwater aquaculture as the biofloc candidates potential development. This study was done through the water quality assessment and potential indigenous microbes isolation approach. Sampling was retrieved from the water subcomposite and sediment on each intertidal inlet and outlet. The water quality of brackishwater pond and intertidal swamp tended to lack of nutrient as containing inoptimal dissolved oxygen, salinity, pH, and ammonia content for the milkfish culture. Therefore, liming and fertilizing should be done to enrich the indigenous microbes. Eucaryote microbe isolated from the enrichment media was the aquatic fungi (multicellular and unicellular fungi) and microalgae (bacillariophyta, chlorophyta, and cyanophyta). Fungi and yeast formed a flocculation with microalgae. This form will become a biofloc candidate as a live feed and water quality controller for the development of brackishwater aquaculture in the swamp area.

Keywords: eucaryote microbes, biofloc, aquaculture, pond, brackish water swamp

ABSTRAK

Mikrob eukariot berperan penting dalam pembentukan bioflok di tambak budidaya pada lahan rawa payau. Bioflok menjadi pakan alami untuk budidaya ikan bandeng atau udang. Tujuan riset ini adalah mengidentifikasi mikrob eukariot rawa payau potensial untuk pengembangan kandidat bioflok di akuakultur rawa payau. Riset ini dilakukan dengan pendekatan kualitas air serta isolasi mikrob *indigenous* tambak dan rawa pasang surut untuk mendapatkan isolat mikrob potensial sebagai kandidat pembentuk bioflok. Sampling dilakukan secara subkomposit pada air dan sedimen di setiap *inlet* dan *outlet* pasang surut. Kualitas air tambak dan rawa pasang surut menunjukkan kondisi oksigen terlarut, salinitas, pH, dan amonia berada di luar batas optimum budidaya ikan bandeng, sehingga perlu dilakukan pengapuran dan pemupukan untuk memperbanyak mikrob *indigenous*-nya. Isolasi mikrob eukariot dari media pengayaan diperoleh jenis fungi akuatik yang multiseluler dan uniseluler, sedangkan mikroalga yang diperoleh yaitu dari golongan Bacillariophyta, Chlorophyta, dan Cyanophyta. Fungi yang dapat membentuk hifa dapat merangkai mikroalga dan khamir untuk membentuk flok. Susunan ini diharapkan dapat menjadi bahan bioflok yang berguna sebagai pengendali kualitas air sekaligus pakan alami bagi pengembangan budidaya di lahan rawa payau.

Kata kunci: mikrob eukariot, bioflok, budidaya, tambak, rawa payau

INTRODUCTION

Brackishwater aquaculture is shrimp and fish culture in the pond using brackishwater applied at about 200,000 ha along with the coastal areas (Kumaran *et al.*, 2018). The brackish wetland is usually a swamp that has a low fertility rate and pH level, making it less suitable for agricultural development. The development of aquaculture is expected to increase this land productivity and improve the local people welfare. The potential utilization of swamp water is still great for the brackishwater aquaculture industry. The development of aquaculture on the intertidal brackish land continues to be carried out mainly for the enhancement of marginal land productivity and achieving the fish culture production target.

The natural productivity of the swamp water becomes one of the important parts of the development of the swamp aquaculture. Determining the level of natural productivity usually depends on the water primary productivity, namely phytoplankton. Phytoplankton comes from in situ microalgae that will symbiotically relate to other aquatic microbes to form a basic food chain in the brackish wetland (Madigan *et al.*, 2015). The microbial community is a key player in the nutrient cycle process and maintenance, as well as the fish health in the aquaculture system. The composition and function of eukaryote microbes in the aquaculture system is still much unknown (Boaventura *et al.*, 2018). Therefore, microbial abundance can be used as an ecological approach in the sustainable development of sustainable swamp aquaculture. The swamp fish culture has much been done by the restation system without water exchange. One method for the fish and shrimp culture intensification without the water exchange can be done by the biofloc development (effective microbial groups). This makes the aquaculture activities more sustainable in terms of production as it maintains water quality, efficient feed, and culture biota health (Martinez-Porchas & Martinez-Cordova, 2012).

The aquatic microorganisms play an important role in the water ecosystem and biogeochemical cycles (De Melo *et al.*, 2018). The eukaryote microbes, comprising microalgae, fungi, and microzooplankton, are important components of water productivity. These organisms combine themselves into the water mutualism symbiosis. The aquatic lichene has been found in the brackishwater as well as the terrestrial area. Lychene is a symbiosis between fungi and

photosynthetic algae or cyanobacteria. This symbiosis is the biofloc starter applied to shrimp, milkfish, and fish ponds. The microbial plankton community forming flocs becomes is important in the pelagic food nets (Easson & Lopez, 2019).

The brackishwater quality is beneficial for cultured fish growth and survival (Venkatachalam *et al.*, 2018). The aquatic microbial flocculation can be a simple technology to control the water quality, feed, even disease prevention (Crab *et al.*, 2012). The successful application of bioflocs for sustainable aquaculture development is determined by the nutrient availability, one of which is the C and N ratio in the culture medium (Pérez-Fuentes *et al.*, 2016). These nutrients are utilized to proliferate microbial inoculants given in the culture media. The in situ microbes given nutrients will develop according to the ecosystem requirement or depending on the microbial composition in the ecosystem. This makes the microbial candidate isolation is necessary to be utilized as Bioflocs former based on the condition of land and water quality during the aquaculture activities. This study aimed to identify the potential of the eukaryotic microbes of swamp area for the biofloc candidate development in the swamp culture.

MATERIALS AND METHODS

This study comprised four steps, namely sampling, cultivation-enrichment, observation, and microscopic identification of the eukaryote microbes.

Sampling

Sampling was performed on November–December 2016 in the swamp area of Tanjung Lago Village, Banyuasin, South Sumatra. The water samples were obtained from four locations (two locations from the brackishwater culture ponds, and two other locations from swamp water areas). Two sampling points represented the intertidal inlet and outlet on each location subcompositely. The water samples were obtained for further water quality and microbial isolation. The in situ water samples were analyzed based on the quality through the temperature, brightness, pH, salinity, dissolved oxygen, and total dissolved solid parameters. Meanwhile, water samples for the other water parameter analysis (BOD, COD, ammonia, nitrate, nitrite, phosphate, Pb, Hg, and Cd) were kept on the refrigerator. The water sample for the microbial isolation was filtered

using a plankton net and kept cool in the bottle at the refrigerator temperature (Addo *et al.*, 2016; Madigan *et al.*, 2020).

Enrichment and cultivation

Water samples of 200 mL (made with two replications) on each location were moved into the Erlenmeyer. These samples were enriched with the sterilized technical fertilizer (TSP ($\text{Ca}_3(\text{PO}_4)_2$), urea ($\text{CO}(\text{NH}_2)_2$), ZA ($(\text{NH}_4)_2(\text{SO}_4)$, Gardasil B) at pH 6–7 under a tube lamp with 500 – 1000 lux. Agitation was performed through aeration for 24 hours, then shaken at 3 times a day for 1 minute every 08.00, 12.00, and 16.00 (GMT+7) until all samples were ready to be identified and isolated (7–14 days). The fungi cultivation was performed using potato dextrose agar (PDA) from the diluted (10^{-4}) enriched isolate using physiological NaCl and grown for 5 days. and shook 3 times a day.

Observation and identification

The eukaryote microbial identification containing phytoplankton and zooplankton was performed using a microscope with 40 and 100 times magnification and assisted with the identification book of Davis (1955). The fungi identification was performed until the proposed groups from the multicellular and unicellular based on the morphological observation. The morphological difference was observed from the specific characteristics of hyphae cells (Madigan *et al.*, 2020).

Data analysis

The observational data of eukaryote microbial types were analyzed descriptively along with the percentage of relative abundance on each plankton type and uniformity on each habitat (swamp or pond). The water quality measurement data result were also analyzed descriptively using the dendrogram analysis (Minitab 14 program) and

grouped based on the uniformity level between the eukaryote microbial habitats.

RESULTS AND DISCUSSION

The swamp water and sediment sampling on the intertidal area was performed during the low-tide reaching the up-tide. Sampling was performed on two areas representing milkfish/shrimp culture ponds (T.I and T.II) and intertidal swamp areas (R.I and R.II) (Figure 1a and 1b). Samples were taken from two points representing the intertidal inlet and outlet. The inlet point was closed to the water entrance from the river, while the outlet point was closed to the seawater way (based on the freshwater flow).

The data obtained from the first sampling (in situ) is shown in Table 1. The water quality result indicates that the intertidal swamp areas have a higher pH tendency than the common water. This is because there was a liming condition during the culture preparation process. High pH level tendency from acid to neutral will be followed with the high microbial diversity in the water (Wijayanti *et al.*, 2019), improving the opportunity to obtain the prospective microbial isolates to maintain the water quality, specifically when utilized as the fish swamp culture water source.

The water quality was still at the range of the national standard for brackish water aquaculture shrimp and milkfish), as the brightness was 30–40 cm and nitrite content was <3 mg/L (SNI 6418.3, 2013; WWF, 2014). Some parameters were outside the optimum range of the national standard, as the dissolved oxygen should be 4–8 mg/L, ammonia should be <0.01 mg/L, total organic matter should be 20–25 mg/L, pH should be 7.5–8.5, and salinity should be 5–25 g/L (SNI 6418.3, 2013; WWF, 2014). The salinity level in location 1 was less than the optimum salinity range



Figure 1. Area of swamp culture water of milkfish and shrimp pond (a), and intertidal swamp water area (b). Note: inlet ●, outlet flow ●

Table 1. The water quality of swamp pond (T) and intertidal swamp (R)

No.	Parameter	Unit	Location							
			T.I.1	T.I.2	T.II.1	T.II.2	R.I.1	R.I.2	R.II.1	R.II.2
1	Depth	cm	58.00	61.00	50.00	58.00	51.00	151.00	72.00	60.00
2	Salinity	g/L	2	1	12	18	20	9	11	19
3	Temperature	°C	33.4	34.3	33.8	34.4	33.5	32.5	30.6	30.6
4	pH	unit	7.33	7.17	7.39	7.56	6.68	7.06	6.82	6.85
5	Brightness	cm	46.00	48.75	41.00	36.50	48.00	59.00	61.25	42.00
6	TDS	g/L	3.26	3.25	>10	>10	>10	4.53	>10	>10
7	DHL	ms	5.2	5.6	19.8	19.50	>20	7.9	19.1	>20
8	DO	mg/L	3	2.9	2.8	2.4	2.6	3	2.8	2.8
9	BOD ₅	mg/L	0.9	2.4	1.8	1.9	0.7	2.3	0.9	1.5
10	COD	mg/L	8	9	6	8	9	7	10	7
11	Ammonia	mg/L	0.09	0.12	0.14	0.19	0.33	0.26	0.24	0.19
12	Nitrate	mg/L	0.06	0.09	0.09	0.01	0.14	0.10	0.09	0.08
13	Nitrite	mg/L	0.019	0.021	0.018	0.019	0.024	0.027	0.021	0.018
14	Phosphate	mg/L	0.12	0.12	0.09	0.09	0.01	0.01	0.04	0.04
15	TOC	mg/L	26.3	26.3	27.8	27.8	26.6	26.6	19.3	19.3
16	Pb	mg/L	<0.003	<0.003	<0.003	<0.003	0.020	0.020	0.020	0.020
17	Hg	mg/L	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
18	Cd	mg/L	<0.0015	<0.0015	<0.0015	<0.0015	<0.0015	<0.0015	<0.0015	<0.0015

Note: T.I.1 = Inlet pond 1, T.I.2 = Outlet pond 1, T.II.1 = Inlet pond 2, T.II.2 = Outlet pond 2; R.I.1 = Inlet swamp 1, R.I.2 = Outlet Swamp 1, R.II.1 = Inlet swamp 2, R.II.2 = Outlet swamp 2; 1 and 2 are the replication codes representing the habitat

for milkfish culture. The dissolved oxygen in ponds and swamps were relatively low. The pH value was also averagely low from the optimum range. The ammonia value both in ponds and swamps brackish was still higher than the maximum limit for milkfish culture. Thereby, the water quality system needs to be improved to customize with the water quality standard for milkfish culture. The aeration system improvement, liming, and seawater flow addition can increase the dissolved oxygen, reduce unionized ammonia level, besides increasing the pH and salinity level based on the optimum condition of milkfish culture.

The water quality condition improvement can be done by liming combined with fertilization to increase the swamp microbes potential as probiotics that can improve and maintain the water quality and cultured biota. The swamp microbes containing bacteria, fungi, and phytoplankton are expected to be the microbial candidates as the live feed material for bioflocs. Swamp bacteria symbiotic with fungi can help maintain the water quality by reducing the sulfate, nitrite-ammonium (denitrifier and anammox), and dissolved carbon in the swamp water including the dark water area

(Holland *et al.*, 2012). Phytoplankton that grows in the swamp bioflocs has a role to supply the dissolved oxygen through photosynthesis.

The group analysis result on the water quality data presented as a Dendrogram (Figure 2) indicates that among the four sampling locations (T I, T II, R I, and R II) have no water quality condition difference. This was shown from the water uniformity level among locations that were still above 95%, which means that the swamp water has no different from the pond water. The water quality in pond 2 (TII) and swamp water (RII and RI) were separated from pond 1 (TI). This indicates that the water quality in pond 2 was still closely similar to the swamp water. This also means that pond 2 has not been given much intensive treatment for the cultured biota. In contrast, the water quality has been improved more in pond 1, although there was no significant difference with pond 2 and swamp water. Similar water quality condition is suspectively due to the swamp water as the cultured ponds were still semi-intensive as swamp ponds are usually used for semi-intensive culture of milkfish and shrimp with unmeasured feeding and only relying on the live feed.

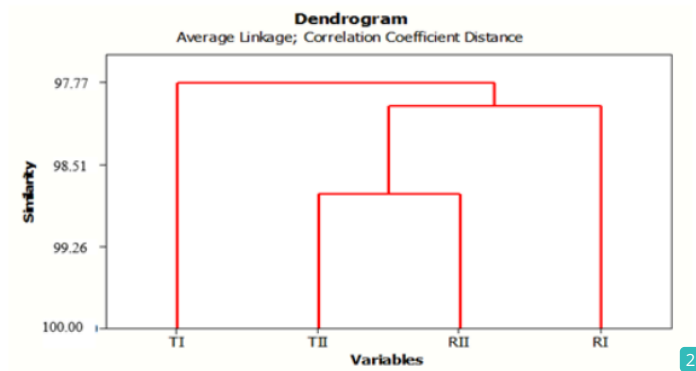


Figure 2. The pond water quality on ponds (T) and swamp areas (R) using Minitab 14. Note : T.I : Pond 1, T.II : Pond 2 ; R.I : swamp 1, R.II : swamp 2; 1 and 2 are the replication codes representing the habitat.

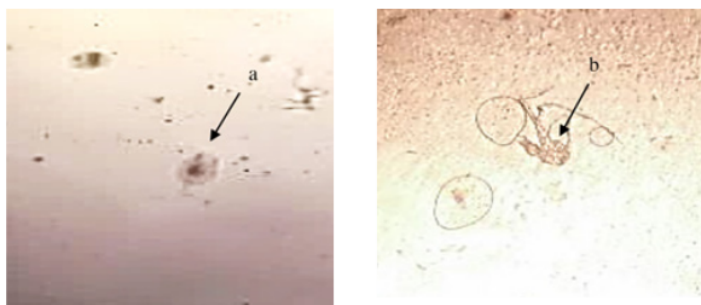


Figure 3. Microzooplankton observation and identification using a microscope with 40x magnification (a. *Brachionus* ; b. Crustacea).

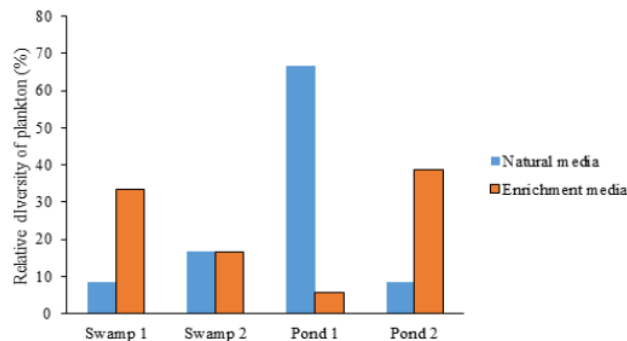


Figure 4. The relative percentage of plankton diversity on different locations

Microzooplankton, such as *Brachionus* and Crustacean type (Figure 3) contained on this swamp water can be utilized as the culture biota live feed, specifically during the larval phase.

The relative plankton diversity from the total identified plankton on each location was compared to the total identified plankton type. The relative plankton diversity was obtained from the ratio of plankton type found on the media and the total identified plankton type on all locations. The relative plankton diversity per location compared

among the different media and enrichment can be seen in Figure 4.

The lack of plankton type found in the samples can be caused by a less fertilized swamp of and dissolved oxygen reduction due to the high organic matter decomposition. Pond and swamp conditions with little or without water exchange will reduce the phytoplankton nutrients and dissolved oxygen level. In addition, to become the natural aerator that enhances the DO in the pond, the water exchange will provide the nutrient

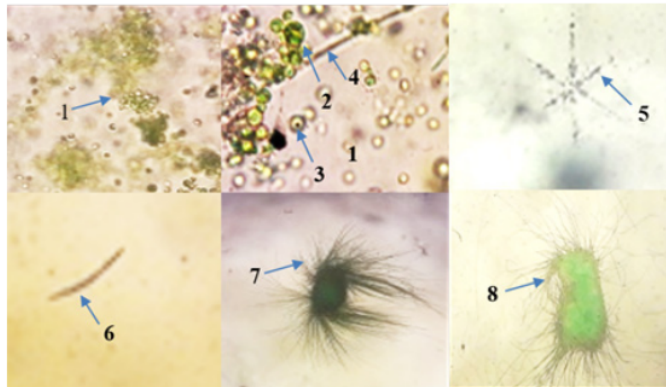


Figure 5. The microalgae identification from water pond sample after enrichment. Note: Phytoplankton types and the magnification: 1. *Chlorella* (40x), 2. *Gleocapsa* (100x), 3. *Aphanotece* (100x), 4. *Oscillatoria* (100x), 5. *Asterionella* (100x), 6. *Thalassiothrix* (100x), 7. *Golenkinia* (100x), 8. *Franccia* (100x).

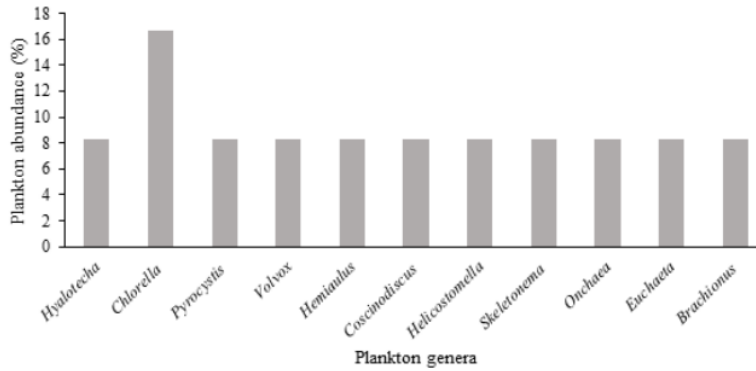


Figure 6. Abundance of plankton in natural habitat medium

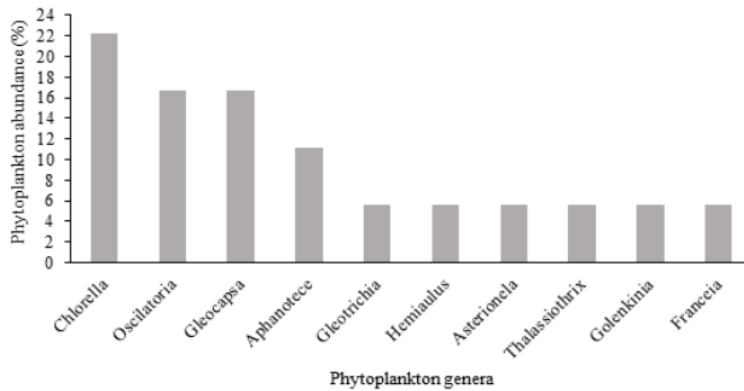


Figure 7. Abundance of phytoplankton in enrichment medium

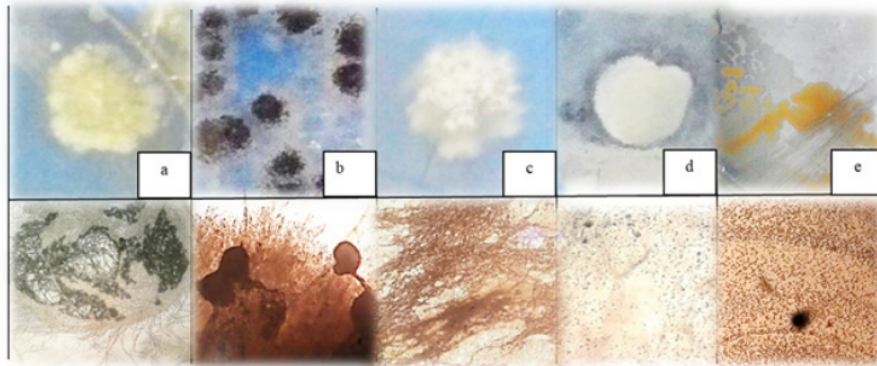


Figure 8. The fungi identification after enrichment with 10x (above) and 40x (below) magnification. Above column shows the colony, while the below column shows the cell (a: cream multicellular, b: black multicellular, c: white multicellular, d: white unicellular, e: yellow unicellular).

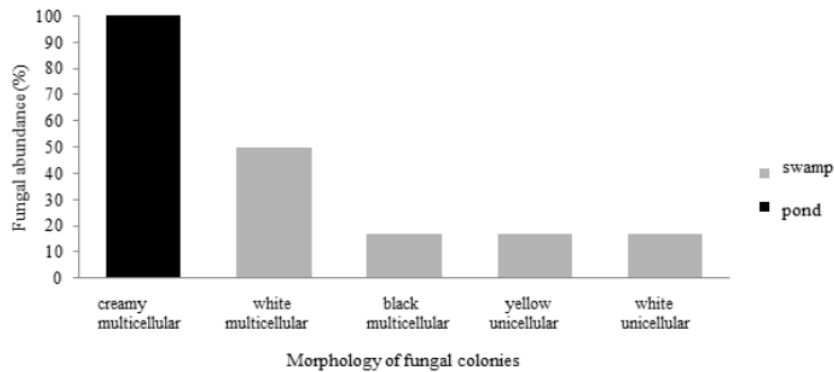


Figure 9. The fungal abundance on each habitat from swamp and pond

required for the phytoplankton photosynthesis in the pond (Cui & Chui, 2017). The water quality of pond and swamp shows poor nutrient conditions, requiring fertilization to multiply the original microbes. The swamp water fertility of swamp ponds can be enhanced through the temporal water exchange from swamp forest and fertilization effort.

The microalgae (phytoplankton) identification was performed after the enrichment by adding microalgae, then aerated and agitated on the media for 7–14 days. The identification result indicates an increased microalgal quantity, although a reduction quantity occurs in the type that grew on technical culture media (Figure 5).

The identified microalgae contained on the enrichment media during the observation period were almost similar to those found in the natural

habitat, dominated by *Chlorella*. The group analysis on pond 1 showed the farthest water quality uniformity from others. This may be caused by the role of freshwater when the rainwater was more dominant than seawater intrusion as seen on low salinity value (1 and 2 g/L) compared to the water salinity level on other locations (<9 g/L). The electrical power also shows similar condition with salinity. *Chlorella* is the easiest growing type of phytoplankton/microalgae with broad salinity tolerance. *Chlorella autotrophica* or *Chlorella* sp. is a type of marine *Chlorella* that can adapt to the freshwater because of its euryhaline capability (Mansfeldt *et al.*, 2016) This type of microalgae can be used as green water on the aquaculture system and live feed for zooplankton and shrimp. *Chlorella* can be an immune-boosting feed for post shrimp larvae with increased prophenoloxidase

activity and total haematocyte, resisting *A. hydrophila* (Maliwat *et al.*, 2016).

The plankton abundance in the origin and enrichment media shows different composition types (Figures 6 and 7). Some phytoplankton and zooplankton were still unable to be preserved while cultured on the enrichment medium. Conversely, there were phytoplanktons growing on the enrichment media, although unable to grow on the original media as stated in Figures 6 and 7. This can be due to the different conformity of enrichment media for different phytoplankton types.

Chlorella dominated twice on the origin habitat media compared to other types (Figure 6). This happened as *Chlorella* can easily grow either in freshwater or brackishwater. High salinity level in the brackishwater induces lipid formation in *Chlorella* cells (Kakarla *et al.*, 2018), making *Chlorella* retain the osmoregulation process with the lipid protection on the cell walls.

The highest phytoplankton abundance on the enrichment media consecutively is *Chlorella*, *Oscillatoria*, *Gleocapsa*, dan *Aphanotece* (Figure 7). *Chlorella* was still in the highest abundance on the enrichment media containing the anorganic technical fertilizer solution often utilized as the fertilizer media for cultivating *Chlorella*. This microalgae type is easily adapted to the various media from the fertilizer until the paper factory and aquaculture sewage media (Daneshvar *et al.*, 2018).

Chlorophyta and Cyanophyta became the easiest group to be grown on the enrichment culture media (Figures 6 and 7). The microalgae identification after enrichment with the technical culture media improved these two groups quantity due to the different element requirement among the two groups and Bacillariophyta, specifically silicate element. Chlorophyta and Cyanophyta are unable to grow well in the media containing silicate instead of Bacillariophyta. The Bacillariophyta or diatoms desperately need silicate elements as the body structure constituent. This makes one of the determinant factors of this microalgae growth. According to Tilman *et al.* (1982), green and blue algae (Cyanophyta) will grow dominantly in the water when the phosphorus-silicate ratio (Si:P) is low, and nitrogen-phosphorus ratio (N:P) is high, and vice versa on the diatoms. In the natural habitat, nutrients and water surface fluctuation⁴⁴ the major factors affecting the phytoplankton composition, while water temperature and rainfall

are very influential on Cyanophyta biomass (Yang *et al.*, 2017). Nutrients affect the fluctuating phytoplankton composition based on the habits and nutrient utilization capability. Water surface fluctuation can affect the passive movement of phytoplankton, changing the composition type, while water temperature and rainfall can affect the Cyanophyta biomass as temperature and rainfall determine the water column height and pH level, making the Cyanophyta near the waterbase or substrate are more exposed to the sunlight during the low tide with the pH level was more than 7 (Issa *et al.*, 2014). Some Cyanophyta such as *Anabaena*, *Oscillatoria*, *Microcystis*, and *Nostoc* in the nitrogen fixation process can be more functional when decreased nitrogen levels occurred in the water column (Issa *et al.*, 2014).

Fungi that grew on PDA cultivation media were multicellular and unicellular fungi either from swamp or pond (Figure 8). The various types of fungi can live in the water habitat by forming layers attached to the substrate or sediment or forming floc in the water column. The density of filamentous fungi is higher in sediment than the water column. Although some environmental parameters are not ideal for fungi growth, the fungi can still survive. The fungi genera that can grow in water and mangrove sediments include *Aspergillus*, *Penicillium*, *Cladosporium*, and *Fusarium*. The fungi capabilities can be explored to produce some enzymes for the biotechnology and pharmaceutical industries (Doi *et al.*, 2018).

The microscope observation on fungi grown in the pond was more diverse than in the swamp (Figure 9) without calculating the total fungi colony. One type of fungi was found dominating the swamp media, i.e the cream multicellular fungi colony, while two fungi types were found dominating the pond media, i.e yellow and white unicellular fungi colony. The pond media contains more organic materials from the feed and aquaculture biota metabolism waste, improving more fungi growth.

The cultured organisms, such as shrimps and milkfish, are the main factor that causes the inclusion of endogenous unicellular fungi in this study. This type of unicellular fungi, namely yeast, can be the source of single-cell protein feed, probiotics, and beta-glucan immunostimulatory material for the cultured organisms (Figure 9) (Meena *et al.*, 2012). Multicellular fungi have the potential to produce polymeric substances (EPS) as the microbial binding material for bioflocs and

biofilm formation (47)cho & Guitierrez, 2017). EPS can damage the performance and productivity of aquaculture facilities and contribute to the (32)ck of harmful phytoplankton against the cultured species. However, EPS can also provide a positive influence on the aquaculture activities by increasing the resistance of cultured larvae and remediate the culture waste during the biofloculation process. EPS is even applicable to the industrial biotechnology aquaculture as an antiviral and immunostimulants agent, as well as anti-biofouling agents (Camacho-Chab *et al.*, 2016). Extracellular polysaccharides can induce nonspecific immune mechanisms and disease resistance in fish (Zhang *et al.*, 2019). Fungi grew on the culture dish were still unknown. Branchiomyces are fungi with negative character causing the mass mortality in tilapia pond culture even the dissolved oxygen level is still above 4 mg/L (Abu-Elala *et al.*, 2016). It is important to ensure that the fungi characteristics isolated from the locations were not pathogenic, toxic, or containing the anti-nutrient substances.

The presence of various fungi and microalgae can be used as the biofloc candidates. Microalgae can form flocs with other microbes such as bacteria, fungi or other microalgae as long as there are flocculation agents, such as polysaccharides (Ummalyma *et al.*, 2017). The utilization of microbial biofloculants can be applied as a natural feed in aquaculture when the feed conditions are fulfilled, i.e nonpathogenic, non-toxic, not producing anti-nutrients, and can be consumed by target biota. Both eukaryote microbes with their symbiotic potential to form bioflocs still needs a further study, considering less information regarding the eukaryotic microbial consortium for the environmental, feed, and disease maintenance in the development of efficient and environmentally friendly aquaculture.

The ongoing development of the world's aquaculture demands new strategies and (25)rnatives to achieve sustainable development. Microbial-based systems are one of the most appropriate strategies for achieving (37) sustainable aquaculture. This system is based on the advantages of microbial proliferation, containing the autotrophic or heterotrophic (20)c microorganisms. The use of microbes can recycle and alter the excess nutrients from wastes, dead organisms, unconsumed feed, and various metabolites into microbial biomass, which are further consumed by the cultured organisms (Martinez-Cordova *et al.*, 2014).

CONCLUSION

Microalgae that can be grown on the culture media come from the group of Chlorophyta and Cyanophyta, while the fungi grown on PDA media from the brackishwater ponds and swamp come from the group of multicellular and unicellular fungi. Both eukaryote microbes have the potential as the biofloc formers in the sustainable development of swamp aquaculture.

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