Sains Malaysiana 49(9)(2020): 2035-2042
http://dx.doi.org/10.17576/jsm-2020-4909-01

Cultivation of *Chlorella vulgaris* in Anaerobically Digested Dairy Manure Wastewater (ADDMW) for Protein and Chlorophyll Production

(Pengkulturan *Chlorella vulgaris* dalam Air Buangan Sapi Cernaan Anaerobik (ADDMW) bagi Penghasilan Protein dan Klorofil)

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ABSTRACT

Chlorella vulgaris *is a eukaryotic, unicellular green microalgae that can be harvested as protein source since it can contain protein up to 50% of its dry weight. However, its cultivation is costly due to the price of its growth medium. In this research we used bioslurry or known as anaerobically digested dairy manure wastewater (ADDMW) as growth medium for* C. vulgaris*, since dairy manure is known to contain high nitrogen and phosphorus and its availability relatively abundant in rural areas. The cultivation of* C. vulgaris *in the ADDMW medium was conducted in lab-scale (19 L) photobioreactors. After 14 days the culture was able to produce chlorophyll content of 34.62 µg/mL, and after 28 days was able to produce protein up to 35% dry weight. Moreover,* C. vulgaris *was also able to reduce PO4-P, NH4-N and NO3-N levels in ADDMW by 45.95, 78.24 and 17.38%, respectively.*

Keywords: ADDMW; Chlorella vulgaris*; chlorophyll; phytoremediation; protein*

ABSTRAK

Chlorella vulgaris *adalah mikroalgae bersel tunggal yang memiliki kandungan protein sehingga 50% berat keringnya. Namun kos pengkulturan* C. vulgaris *adalah tinggi kerana medium pengkulturannya yang mahal. Dalam kajian ini, kami menggunakan air buangan sapi cernaan anaerobik daripada proses penghasilan biogas. Pengkulturan* C. vulgaris *dalam medium air buangan sapi cernaan anaerobik (ADDMW) dalam fotobioreaktor (19 L) berskala makmal selama 14 hari dapat menghasilkan* C. vulgaris *dengan kandungan klorofil 34.62 µg/mL dan dalam 28 hari dapat menghasilkan* C. vulgaris *dengan kadar protein 35% berat kering. Selain itu, kultur* C. vulgaris *dalam medium ADDMW dapat menurunkan kadar PO4-P, NH4-N dan NO3-N ADDMW masing-masing sebanyak 45.95, 78.24 dan 17.38%.*

Kata kunci: ADDMW; Chlorella vulgaris*; fitopemulihan; klorofil; protein*

INTRODUCTION

It is estimated that the current protein demand for 7.3 billion inhabitants of the world today is approximately 202 million tonnes globally. This figure could increase due to rapid population growth combined with rising incomes and urbanizing factors (Henchion et al. 2017). Therefore there is a need for searching alternative protein sources, and microalgal *Chlorella vulgaris* is one of them.

C. vulgaris is a eukaryotic, unicellular green microalga that can contain protein up to 50% of its dry weight (Liang et al. 2015). This microalgae is also an excellent producer of chlorophyll, an important product that is widely used in the food, cosmetics and pharmaceutical industries (Amin et al. 2018).

However, the cultivation of *C. vulgaris* requires high operational costs due to the price of the medium. As an alternative for an expensive growth medium, in this research, we cultivate *C. vulgaris* using anaerobically digested dairy manure wastewater (ADDMW) which is rich in nitrogen and phosphorus content (Taufikurahman & Istiqomah 2019; Wang et al. 2010). We aimed to utilize bioslurry from the process of biogas production (ADDMW) as growth media to cultivate *C. vulgaris* which potentially can be extracted to produce protein and chlorophyll.

MATERIALS AND METHODS

PREPARATION OF MICROALGAE INOCULUM AND GROWTH MEDIUM

The inoculum of *Chlorella vulgaris* was obtained from Balai Besar Perikanan Budidaya Air Payau (BBPBAP) Jepara, Central Java. For preparation, *C. vulgaris* was cultivated using commercial Walne medium in 800 mL container, with a 20% initial concentration of *C. vulgaris.* The photoperiodism employed was 16 h of light: 8 h of dark using a timed white LED. An aeration pump with a power of 1 LPM was used for aeration.

The dairy manure was obtained from the Faculty of Animal Husbandry, Padjadjaran University, West Java the ADDMW as a liquid effluent from a biodigester for biogas production. Sterilization was conducted using an autoclave at 121 °C for 15 min. The remaining solid particles were removed before being utilized as a growth medium.

CULTIVATION OF *Chlorella vulgaris* AND DEVELOPMENT OF GROWTH CURVE

Culture of *C. vulgaris* was transferred to 19-L photobioreactor after its concentration reached a minimum of 105 cells/mL. There were 3 variations on the growth medium we used in this study: The Walne medium which contain trace metal solution, vitamin solution and nutrient solution, with a concentration of 1 ppm (*v/v*), ADDMW, and a mix of both media.

A growth curve was made based on the cell number data to find out the growth phase of *C. vulgaris*. The cell number's measurement was carried using haemacytometer method with at four replications. A trinocular microscope integrated with NIS-Elements D software version 4.00 was also employed.

EXTRACTION AND DETERMINATION OF PROTEIN AND CHLOROPHYLL CONTENT IN *C. vulgaris*

Protein extraction was carried out using 3 mL of 0.5 N NaOH in two stages. Each stage lasts for 10 min at 80 °C. The supernatant obtained from this process was analyzed using the Bradford method to determine protein content of *Chlorella*. The wavelength used for the UV-VIS spectrophotometer was 595 nm with the BSA standard curve ranging from 0 to 1000 ppm. The determination of protein content was given in (1).

$$\%\ \text{protein} = c\ x\ \frac{Vf}{1000} x \frac{1}{DW} \quad (1)$$

with c is protein content in *C. vulgaris* (ppm); Vf is filtrate volume from 2 stages extraction (mL); and DW is dry weight (grams).

Chlorophyll extraction was carried out using the maceration method with acetone solvent and no light for 24 h (Simon & Hellineell 1998). The analysis of chlorophyll content was held using a UV-VIS spectrophotometer with a wavelength of 645 and 663 nm. Chlorophyll concentration was calculated using the following equations (2, 3, 4) (Zheng et al. 2011).

$$\text{Chl a } (\mu g/mL) \approx 11.8668\ A_{663} - 1.7858\ A_{645} \quad (2)$$

$$\text{Chl b } (\mu g/mL) \approx 18.9775\ A_{645} - 4.8950\ A_{663} \quad (3)$$

$$\text{Total chlorophyll } (\mu g/mL) = \text{Chl a} + \text{Chl b} \quad (4)$$

DETERMINATION OF PO4-P, NH4-N, AND NO3-N LEVELS IN THE MEDIUM

Prior to phosphorus and nitrogen analysis, the culture was centrifuged at 5000 rpm for 10 min. The determination of orthophosphate (PO4-P), ammonium (NH4-N), and nitrate (NO3-N) content were carried under a UV-VIS spectrophotometer with each specific reagent and specific wavelength.

PO4-P was analyzed using the Stannous Chloride ($SnCl_2$) method with a wavelength of 660 nm. NH4-N was analyzed using Nessler with 420 nm of wavelength, while NO3-N was analyzed using APHA-4500-NO_3-B-2012 standard using HCl as a reagent with 220 and 275 nm as the wavelengths. The determination of removal efficiency was given in (5).

$$\eta_a = \frac{(c_{at} - c_{a0})}{c_{at}} x100\% \quad (5)$$

with η_a is the removal efficiency of substance a; c_{at} is the concentration of substance a at time-t; and c_{a0} is the concentration of substance a at time 0.

RESULTS AND DISCUSSION

PROTEIN PRODUCTION BY *C. vulgaris* IN WASTEWATER MEDIUM

Cultivation of *C. vulgaris* for 14 and 28 days was carried out to see the comparison of product acquisition in the form of protein and chlorophyll in each medium. Cultivation in ADDMW medium for 28 days, has resulted

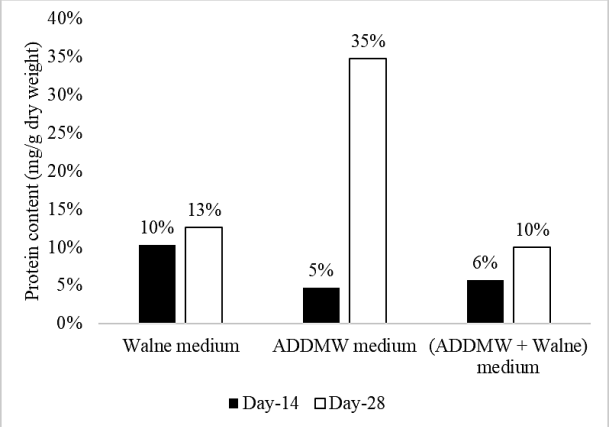

FIGURE 1. The comparison of protein content in C. vulgaris in various medium

C. vulgaris to produce protein content 35% which is the highest compared to when grown in other media (Figure 1). This value is much higher than the protein content obtained from the other mediums that contain Walne. This indicated that the addition of Walne into the waste medium could not help to increase the level of protein in *C. vulgaris* significantly. However, the protein content obtained from this study was still relatively low compared to the previous studies, which was able to produce the protein levels in the range of 45 up to 60% of its dry weight (Chinnasamy et al. 2009; Hu et al. 2013; Jian-Ming et al. 2010).

The decreased levels of protein in microalgae may lead to a significant increase in carbohydrate and lipid levels, simultaneously. This may be caused by the interconnection of biosynthetic pathways that produce these three biochemical compounds due to energy allocations (Jian-Ming et al. 2010). Meanwhile, other research showed that cultivated *Chlorella* sp. in the ADDMW medium for 21 days was only able to produce lipids in the range of 9.0-13.7% of their dry weight (Wang et al. 2010).

Furthermore, the carbon fixation in the photosynthesis of microalgae is strongly influenced by the nitrogen and phosphorus composition of the substrate medium. It will tend to produce carbohydrates rather than protein if the culture medium has a limited nitrogen content (Hena et al. 2015), which can be represented by the N/P ratio values below 5 (Goncalves et al. 2017). Since the estimated N/P ratio in all mediums during the cultivation period was recorded below than 5 (Table 1), the cultivation was operated in nitrogen-limiting condition.

TABLE 1. Estimated N/P ratio during cultivation in all medium variations

Day	Estimated N/P ratio in the medium		
	Walne	ADDMW	ADDMW + Walne
0	0.00	0.98	0.54
4	0.53	3.14	1.43
8	0.67	3.33	0.65
12	1.44	2.27	1.21
14	1.92	1.30	0.41

CHLOROPHYLL PRODUCTION BY *C. vulgaris* IN WASTEWATER MEDIUM

After 14 days cultivation, chlorophyll content in *C. vulgaris* grown in ADDMW was slightly higher than chlorophyll content in *C. vulgaris* grown in Walne medium (Figure 2). After 28 days however chlorophyll content of *C. vulgaris* grown in ADDMW reduced significantly, while chlorophyll content in *C. vulgaris* grown in Walne medium was relatively still the same as at 14 days. The highest total chlorophyll content in this study was depicted at 65.39 µg/mL, which was obtained from the Walne medium after 28 days. *Chl*-a content in *C. vulgaris* after 14 days with a concentration of 34.62 µg/mL was about 3 times higher than the *Chl*-a value obtained by the Zehnder medium (Seyfabadi et al. 2011), but lower when compared to the ones obtained from a synthetic waste medium, which was able to reach up to 60 µg/mL (Zhu et al. 2018).

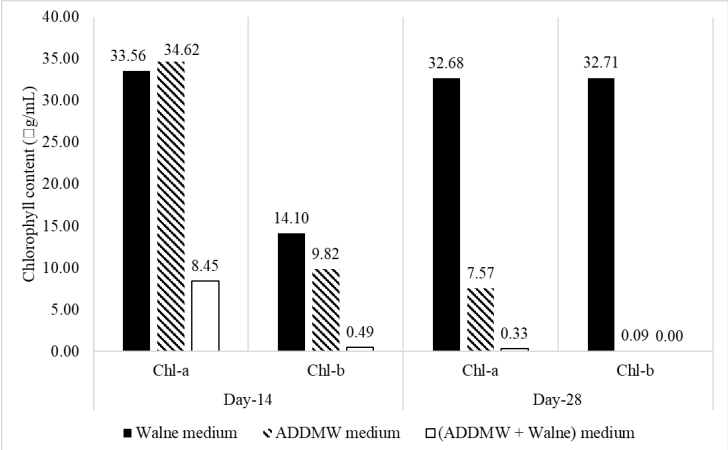

FIGURE 2. The comparison of *Chl*-a and *Chl*-b content in *C. vulgaris* cultivated in various medium

The differences in *Chl*-a value among the studies were probably caused by differences in intensity, type, and a regime of light provided. The difference could also occur because of different nitrogen composition in the medium, which affect the synthesis process of the chlorophyll (Safafar et al. 2016).

Furthermore, the adequate levels of chlorophyll found in the ADDMW medium suggest the potential of this medium to produce chlorophyll as a by-product of the phytoremediation process. Chlorophyll production could be carried out before the lipid extraction process to produce an efficient and profitable process (Zheng et al. 2011).

GROWTH KINETICS OF *C. vulgaris* IN VARIOUS MEDIUM

The growth curves based on cell count data showed that *C. vulgaris* successfully entered the exponential growth phase starting on the 12th day of cultivation (Figure 3). The ADDMW medium can generate the largest number of *C. vulgaris* cells up to 2.73×10^6 cells/mL, followed by the Walne medium with 1.69×10^6 cells/mL, and the ADDMW + Walne medium which presents the fewest number of cells with 3.03×10^5 cells/mL.

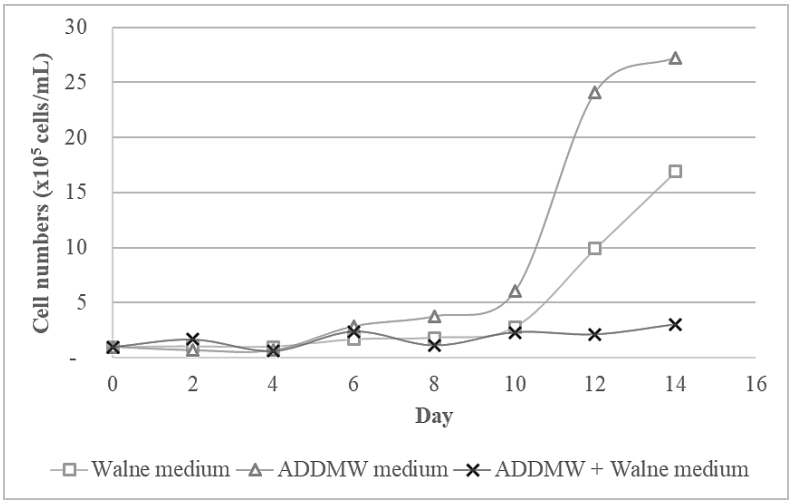

FIGURE 3. The growth curve of *C. vulgaris* on various medium based on cell count data using haemacytometer

The highest growth kinetics of *C. vulgaris* on the ADDMW medium is also reflected by the higher specific growth rate (μ) values and shorter doubling time (*dt*) (Table 2). The μ value of *C. vulgaris* cultivated on the ADDMW medium was 0.693 $days^{-1}$, 53% higher than the Walne medium, and 97% higher than the ADDMW + Walne medium. This value is also higher than the μ value from other studies which reached 0.409 day^{-1} with similar operating conditions (Wang et al. 2010). However, the value is lower than the one obtained at optimal operating conditions on a smaller scale which reached 1.00 $days^{-1}$ (Ji et al. 2014). Therefore, specific growth rate values are the best way to explain the ability of a species in adapting to its environmental conditions (Hena et al. 2015). Thus, the low value of specific growth rates in the ADDMW + Walne medium indicates the difficulty of *C. vulgaris* adaptation to that medium.

TABLE 2. Comparison of specific growth rate (μ) and doubling time (*dt*) from *C. vulgaris* cultivated in various medium

Medium	μ (day^{-1})	*dt* (day)
Walne	0.452	1.53
ADDMW	0.693	1.00
ADDMW + Walne	0.352	1.97

This phenomenon can also be described using the N/P ratio. It is the ratio between the total inorganic nitrogen and the total inorganic phosphate available in the microalgae culture medium. This value is significant since it is closely related to the growth kinetics of microalgae (Ji et al. 2014). Although the N/P ratio during cultivation shows a separate trend for each medium (Table 1), which follow the movement of the growth curve of *C. vulgaris* (Figure 3) in the same period. The optimal N/P ratio for algal growth is 6.8-10 (Wang et al. 2009).

REMOVAL EFFICIENCY OF N AND P SUBSTANCE FROM ADDMW

The concentration of orthophosphate (PO4-P) in the ADDMW medium generally shows the declining trend for 14 days cultivation (Figure 4). The initial [PO4-P] in the ADDMW medium was reported at 26.72 ± 0.00 ppm, while the lowest [PO4-P] was obtained on the 12th day with the value of 14.44 ± 3.02 ppm. Thus, the PO4-P removal efficiency from this study was calculated at 45.95%, which is similar achieved by Choi et al. (2012) with a range between 44.80 and 48.76. However, this value is still far below the results attained by other studies that can reach 62% up to 94% (Franchino et al. 2013; Rao et al. 2011; Wang et al. 2010, 2009).

Phosphate compounds themselves are needed by *C. vulgaris* in a small quantity only to synthesize phospholipids, ATP, and nucleic acids (Rao et al. 2011). Nevertheless, *C. vulgaris* was also able to absorb phosphate at high removal efficiency with several

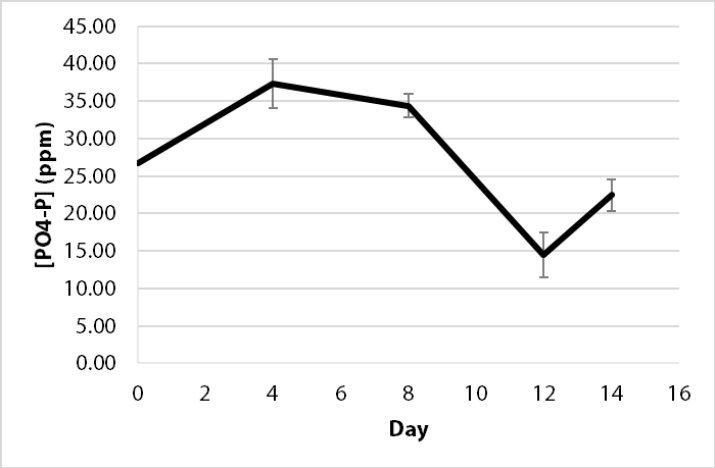

FIGURE 4. The orthophosphate (PO4-P) ion concentration in the ADDMW medium during the cultivation of *C. vulgaris*. Error bar shows the deviation standard with 3 replicates

mechanisms. One of the mechanisms is storing an excess amount of phosphate absorbed into the form of polyphosphate compounds (Powell et al. 2009). Another mechanism is precipitation, which can occur due to increased pH and dissolved oxygen concentrations (Kumar et al. 2018). Conversely, a decrease in pH can lead to decreasing the concentration of dissolved phosphate in the medium (Sayadi et al. 2016).

The ammonium (NH4-N) concentration in the ADDMW medium showed a reduction after a significant

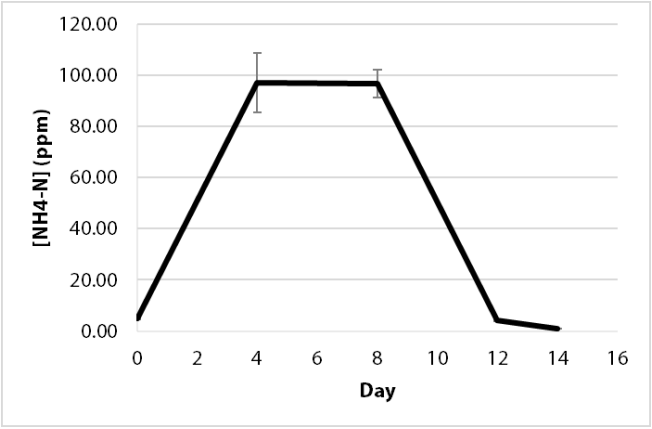

FIGURE 5. The ammonium (NH4-N) ion concentration on the ADDMW medium during the cultivation of *C. vulgaris*. Error bar shows the deviation standard with three replicates

increase on the 4th day (Figure 5). The initial [NH4-N] was depicted at 4.84 ± 0.39 ppm, while the lowest concentration reached at 1.05 ± 0.00 ppm on the 14th day. The value of NH4-N removal efficiency obtained was 78.24%, this is similar to the case reported where NH4-N removal reached 74.70 - 0.00% (Rao et al. 2011; Yang et al. 2015). Ammonium was used by *C. vulgaris* as the main source of nitrogen since it can be directly absorbed by the cell in one stage (6).

$$\text{Glutamat} + NH_4^+ + \text{ATP} \rightarrow \text{Glutamin} + \text{ADP} + \text{Pi} \quad (6)$$

Meanwhile, a significant increase of NH4-N at the 4th day occur because the ammonium ion undergoes a gasification process (Seyfabadi et al. 2011). This gasification is common because NH_4 is a thermodynamically less stable form of nitrogen (Podder & Majumder 2016). This phenomenon may increase the pH since the concentration of ammonium ions and hydroxide ions in the medium has an equilibrium relationship (7).

$$NH_3 + H_2O \; NH_4^+ + OH^- \quad (7)$$

The nitrate (NO3-N) concentration in the ADDMW medium showed an increase trend (Figure 6). Nitrate absorption efficiency values were obtained at 17.38%. This value is lower than expected from the previous work by at least 29% (Wang et al. 2009). The low efficiency of nitrate absorption shows that *C. vulgaris* prioritizes the ammonium rather than nitrate as the nitrogen sources, especially when the ammonium concentration is still quite high. As stated by Goncalves et al. (2017), microalgae first reduce nitrate to nitrite using the nitrate reductase and reducing agent NADPH (8).

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \quad (8)$$

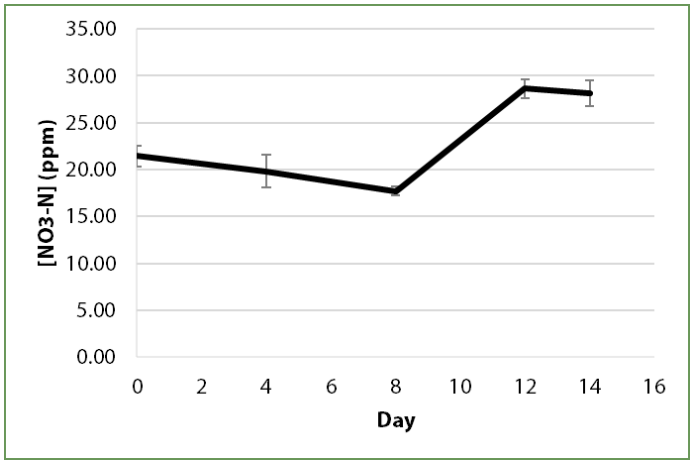

FIGURE 6. The nitrate (NO3-N) ion concentration on the ADDMW medium during the cultivation of *C. vulgaris*. Error bar shows the deviation standard with three replicates

Furthermore, nitrite is transformed into ammonium using the nitrite reductase with a ferredoxin as a reducing agent (9), and ammonium will then be absorbed directly by microalgae (6).

$$NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O \quad (9)$$

CONCLUSION

The cultivation of *Chlorella vulgaris* in the anaerobically digested dairy manure wastewater (ADDMW) medium was able to produce chlorophyll-a 34.62 µg/mL after being cultivated for 14 days and a protein content of 35% after 28 days of cultivation. The addition of Walne to the ADDMW medium could not increase the protein and chlorophyll content of *C. vulgaris*. Moreover, *C. vulgaris* was also able to reduce levels of PO4-P, NH4-N, and NO3-N in the wastewater medium with a removal efficiency of 45.95, 78.24 and 17.38%, respectively. The use of ADDMW for growth media and nutrient source of *C. vulgaris* was able to produce significant amount of biomass, chlorophyll and protein from the microalgae, and at the same time reduce the amount of phosphate, ammonium-nitrogen, and nitrate-nitrogen in organic wastewater from bioslurry.

ACKNOWLEDGEMENTS

We thank The Institute for Research and Community Services of ITB (IRCS/LPPM-ITB) and the Ministry of Education and Culture (Indonesia) for funding this research through P3MI and PDUPT schemes for the year of 2018, 2019 and 2020.

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Received: 15 January 2020
Accepted: 15 April 2020