Enhancement of *C. vulgaris* species growth using aquaculture sludge extracts

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Abstract: High-value microalgae have many useful substances that can be used for many applications. This study established the effect of the sludge extract (SE) on *C. vulgaris* species. Five different autoclave extraction parameters were assessed on SEs, i.e. 1-h at 105 °C, 2-h at 105 °C, 1-h at 121 °C, 2-h at 121 °C, and 24-h at room temperature (natural extraction). The SE obtained from the Sabak Bernam (SB) and Kota Puteri (KP) was supplemented with Conway media and checked using microplate incubation technique. Microalgae cultivation in control (media) and enriched (media + SE) samples were incubated for nine days at 25 °C with 33.75 µmol photons m^{-2} s^{-2} light intensity on a 12-hour light: 12 h dark cycle. *C. vulgaris* (TRG 2A) and *C. vulgaris* (TRG6-B01) showed better growth in modified SE compared to control yet no significance differences ($p > 0.05$) were observed. The specific growth rate (SGR) of *C. vulgaris* (TRG 2A) showed significant differences ($p < 0.05$) between SB and KP SE while, *C. vulgaris* (TRG6-B01) showed no significant differences ($p > 0.05$). The organic matter contents in the SE and autoclave at extended high temperatures influences the microalgae growth.

Keywords: *C. vulgaris*, extraction parameters, sludge extract, specific growth rate.

1. Introduction

Microalgae are a diverse group of autotrophic, unicellular species that are mainly photosynthetic. More than 40,000 eukaryotic microalgae organisms have been investigated as alternative sources of animal feed, nutritional supplements, nutraceuticals and pharmaceuticals in last few decades (Chu, 2012). For a balanced diet, microalgae are regarded as a remarkable but poorly explored natural source as several microalgae organisms are considered to be abundant in carbohydrates, proteins, lipids and nutritionally important constituents (Sathasivam et al., 2019). Furthermore, the worldwide vitality emergency of the 1970s prompted the discovery of algae as inexhaustible and economical wellsprings of biofuel creation, urging microalgae to be investigated as another field of study for fuels and other exceptionally esteemed items (Paul Abishek et al., 2014). The development of algae additionally has developed into new zones, for example, food and feed, biofuels and pharmaceuticals (Khan et al., 2018).

Chlorella vulgaris has indicate potential for many applications by its rapid growth rates, large number of biochemical compounds and adaptability to environmental stresses (Ru et al., 2020). They have ability to develop under heterotrophic and mixotrophic conditions to adapt to a variety of diverse environments (Kong et al., 2020).Nutritional condition for microalgae development is significant as it can influence the development of microalgae. So as to give high algae yields, microalgae need a consistent source of a few inorganic supplements, for example, nitrogen (N), phosphorous (P), potassium (K) (da Silva & Ribeiro, 2019) and iron (Fe), silica (SiO_2) where these components were fundamental for development

of microalgae. Artificial culture medium contains all the fundamental components for microalgae development where by far most of studies utilize synthetic medium which is nourishment enhanced to create microalgae development (Ramaraj et al., 2010). Nevertheless, urban, industrial or agricultural wastewater as a cultivation tool, may be used while at the same time serving as a biological treatment to extract and recover nitrogen and phosphorus from those sources (Hwang et al., 2016). . The integration of the compost medium and inorganic medium could theoretically replace the major nutrients such as nitrate and phosphate needed for the cultivation of microalgae (Chew et al., 2018). This would lead to a more sustainable method of industrial growth of microalgae for subsequent biorefineries.

Aquaculture sludge from two ponds, Sabak Bernam shrimp pond (SB) and Kota Puteri fish pond (KP), were tried in this examination to determine their natural microalgae growth-promoting effects. The aim of this examination was to assess the development impacts of sludge extracts (SEs) on *C. vulgaris* species. More specifically, our point is to assess the potential of SE on high-esteem microalgae growth.

2. Materials and Methods

2.1 Sludge Extracts (SEs)

Sludge was gathered from two sorts of ponds, i.e., SB shrimp pond and KP fish pond. The samples were oven dried at 60 °C for multi week to eliminate dampness and afterward ground utilizing 700 g Swing Type Electric Herbal Powder Grinder (Weifang City, Shandong, China), sieved at 1 mm, and homogenized. The dried sludge samples had been treated with aqueous extraction. Five autoclave extraction treatments were done on the sludge samples: 1-h at 105 °C, 2-h at 105 °C (twice), 1-h at 121 °C, 2-h at 121 °C (twice), and no autoclave, 24-h at room temperature (Arumugam et al., 2020). Allegra-30R axis (Beckman, Indiana, United States) was utilized to centrifuge temperature-treated sludge samples at 700 × g for 15 min. SE's supernatant (around 150 mL) was filtered through a 0.7 µm glass fiber (GF/F, Whatman). The filterated samples were then kept at 4 °C for additional uses. Italics should be used for emphasizing words or phrase in running text, but do not format entire paragraphs in italics.

2.2 Microalgae

Microalgal species used in this study were *Chlorella vulgaris* (TRG 2A) and *C. vulgaris* (TRG6-B01). These microalgae were isolated from Redang and Setiu islands at Terengganu, Malaysia. Conway media was prepared from five basic solutions as described by (Khatoon et al., 2016); mineral solution, trace metal solution, vitamin, silicate and nitrate solution (Arumugam et al., 2020). The microalgae cultures were grown on Conway media under a light intensity of 33.75 µmol photons m^{-2} s^{-1} at 25 ± 0.5 °C on a 12-hour light: 12 h dark cycle. The stock cultures were acclimatised to the experimental conditions before the strains were tested on sludge extracts.

The microplate-incubation technique was performed for the two microalgae utilizing 96-well microplates in the five diverse SE extraction treatments (Fig. 1). The microplate 's border wells were topped off with 200 µL Milli-Q water during the investigation to prevent evaporation. For the rest of the wells, 2nd column (blank) filled with 195 µL of Conway media + 5 µL of 105 °C SE, and the 3rd column loaded up with 175 µL of Conway media + 5 µL of 105 °C SE + 20 µL of microalgae (experiment), as shown in Figure 1 to record the microalgae exponential phase (Arumugam et al., 2020). Similar advances were rehashed in the 4th to 11th columns of a microplate with 105 °C twice (Column 5), 121 °C (Column 7),

121 °C twice (Column 9), and 24 h natural extraction (Column 11). In another microplate, a control experiment was conducted with Conway media without SE on microalgae. The biomass or microalgae growth was estimated by optical density (OD) at 680 nm per 24 h by incubating the microplates for nine days, utilizing the Infinite M200 PRO (Tecan, Austria) microplate reader.

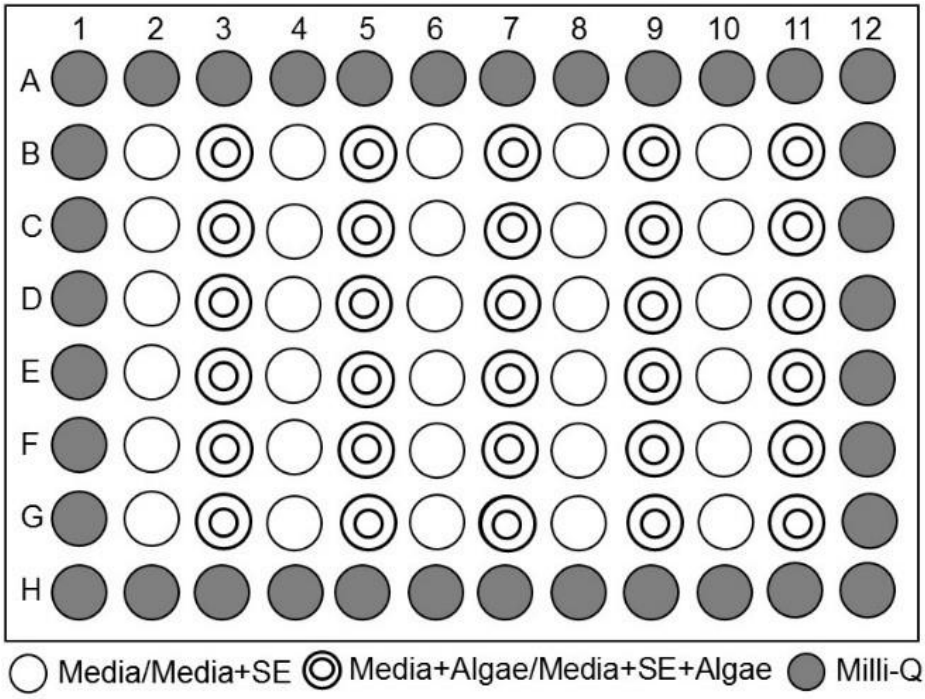

Fig. 1. Microplate-incubation technique of media or media + soil extract (SE) and growth test (media + microalgae or media + SE + microalgae) (Arumugam et al., 2020)

2.3 Data Analysis

Each control and sample has three replicates in a column of microplate. The net optical density (OD) mean value was calculated by deducting OD of control and sample. The specific growth rate (μ) and the division rate (k) of microalgae were estimated as follows,

$$\mu = \ln(N_2\text{-}N_1)/(t_2\text{-}t_1) \qquad (1)$$

$$k = \mu/(\ln 2) \qquad (2)$$

where N_2 and N_1 are the OD at times t_2 and t_1 respectively.

Microalgae growth and maximum OD in the respective SB and KP SE temperature treatment parameters were analysed using independent samples t-test and one-way variance analysis (ANOVA). Significant differences were measured at a confidence interval of 95 % between the various extraction parameters. All statistical analyses were carried out using the IBM SPSS (Statistical Social Science Package) software.

3. Results and Discussion

3.1 Effects of modified SE on *C. vulgaris* growth

Both microalgae species demonstrated positive development patterns under all extraction treatments tested. Fig. 2 (A) showed that the development of *C. vulgaris* (TRG 2A) in all modified SE were higher contrasted with control. It did not show any significant differences ($p > 0.05$) between modified SE and control. This microalga showed higher growth in media + 105 °C twice compared to control and other modified SE (Fig. 2 B) yet, no significant differences ($p > 0.05$) were seen between it. Hence, it showed that modified SE enhances microalgae growth more than control. The *C. vulgaris* sp. obtained the highest total biomass in 100% sludge extract (33.98 ± 0.30106 cells / mL) and good results in TOC (absolute 175 mg / L) and nutrient (Total nitrogen:77.1 %; Total phosphorus: 95.0 %) (Wang et al., 2019).

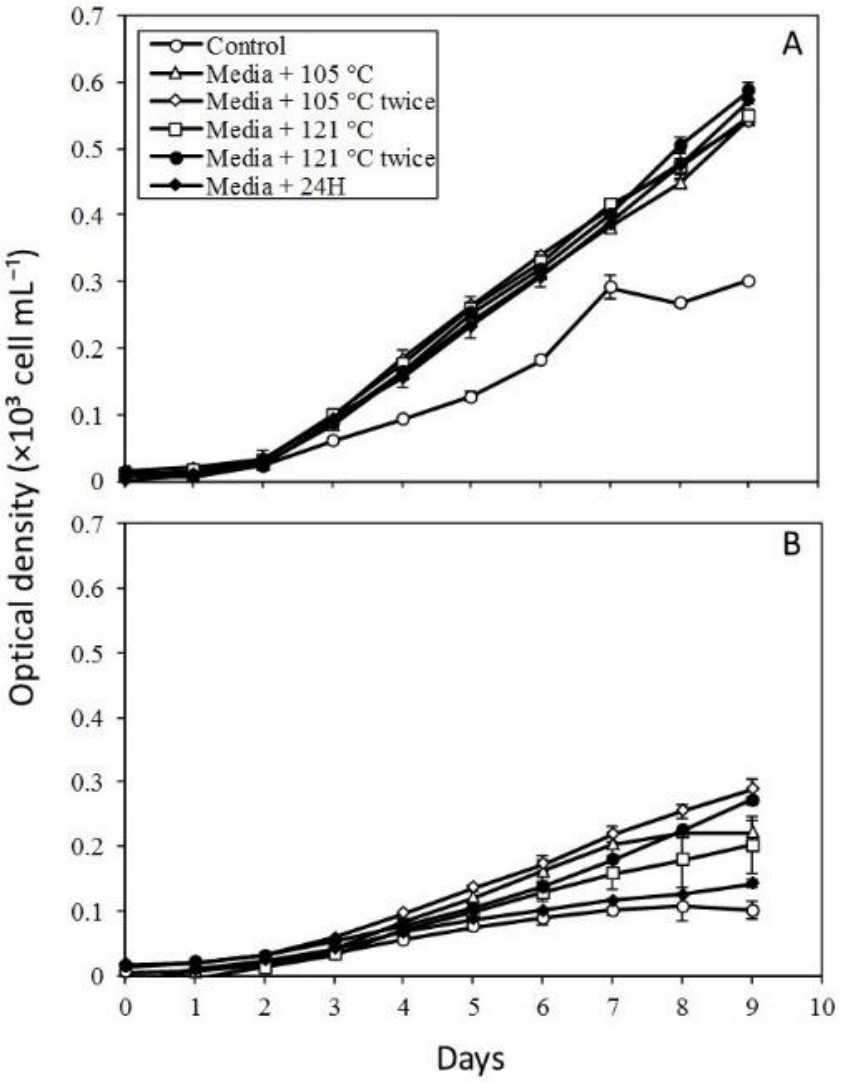

Fig. 2. Optical Density at 680 nm of *C. vulgaris* (TRG 2A) in control, media + 105 °C, media + 105 °C twice, media + 121 °C, media + 121 °C twice and media + 24 hour at (A) SB SE and (B) KP SE. Error bars represent standard deviation (n = 3)

The development of *C. vulgaris* (TRG6-B01) in SB SE indicated higher growth in all modified SE than control (Fig. 3 A). In all the modified SE, media + 105 °C twice shows higher growth until day 8 which was then overtaken by media +121 °C twice on day 9. However, no significant differences ($p > 0.05$) were observed between modified SE and control. In KP SE, the growth was higher in media + 105 °C twice and media + 121 °C contrasted with control (Fig. 3 B) but, it did not show any significant differences ($p > 0.05$) between it. Hence, it is proved that aquaculture sludge extracts have the essential elements needed to maximize microalgae growth. Previous studies include that *C. vulgaris* was cultivated for biomass production and nitrogen removal on certain number of wastewaters like municipal (Lam et al., 2017), industrial (Subramaniyam et al., 2016) and agricultural sewage (Amini et al., 2016). Therefore, it is possible to find promising waste water to replace artificial medium for cultivating algae. If the nutrients in wastewater were well balanced, then it could be an ideal supplement source for algae cultivation (Valverde-Pérez et al., 2015).

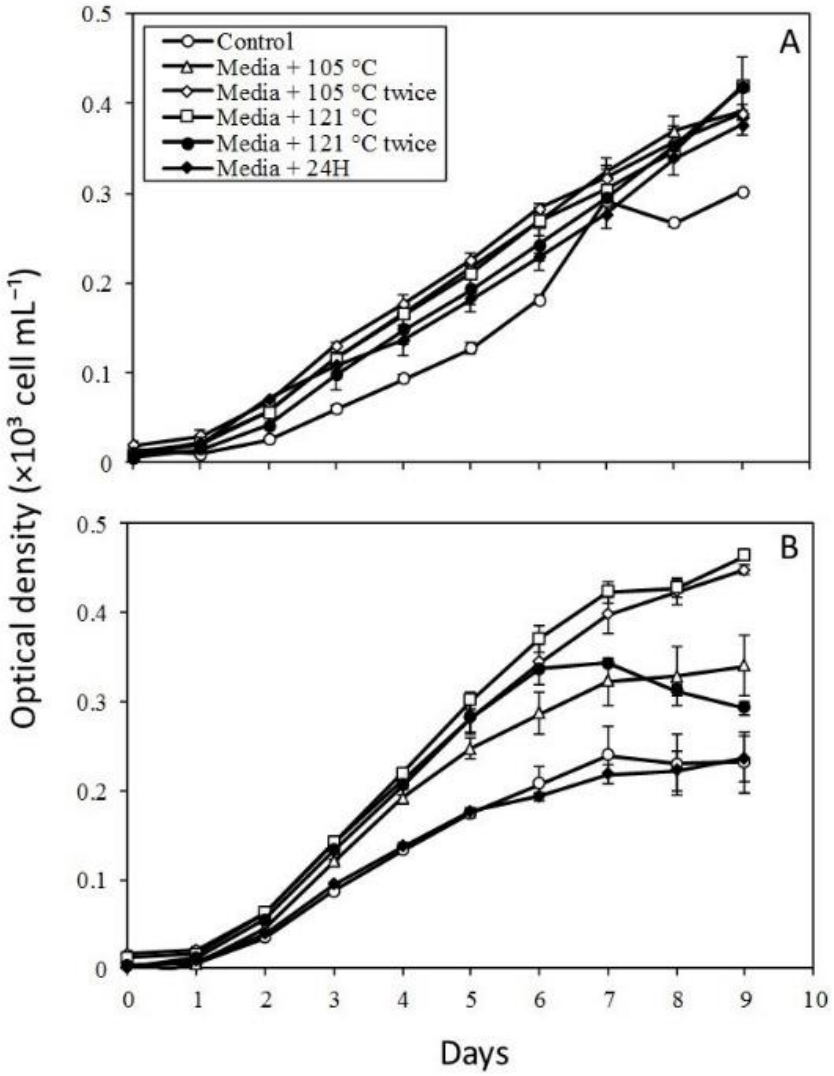

Fig. 3. Optical Density at 680 nm of *C. vulgaris* (TRG 6- B01) in control, media + 105 °C, media + 105 °C twice, media + 121 °C, media + 121 °C twice and media + 24 hour at (A) SB SE and (B) KP SE. Error bars represent standard deviation (n = 3)

3.2 The maximum OD, specific growth rate (SGR) and division rate of *C. vulgaris* species

The maximum OD of the two species were different between modified SE and control (Table 1 and 2). For *C. vulgaris* (TRG 2A), the maximum OD was in media + 121 °C twice in SB SE, while in KP SE, maximum OD was observed in media + 105 °C twice. Maximum OD of *C. vulgaris* (TRG 2A) demonstrated significant differences ($p < 0.05$) between SB and KP SE. The maximum OD of *C. vulgaris* (TRG6-B01) was seen in media + 121 °C and media + 121 °C twice in SB SE, while in KP SE, media + 105 °C twice and media + 121 °C indicated higher OD. However, no significant differences ($p > 0.05$) observed between the SE. The higher nutrient contents in modified SE compared to control makes the microalgae grew well in modified SE. Sánchez-Bayo et al. (2020) noted that the metals in the wastewater serve as important trace elements to promote metabolic growth of microalgae. In this investigation, media + 105 °C twice, media + 121 °C and media + 121 °C twice has the high estimation of maximum OD. This is due to the autoclaved soils showed more dissolved organic matters by subsequent analysis than in untreated soils (Arumugam et al., 2020).

Depending on the media parameterization type and three stages, the SGR (μ) of microalgae species in modified SB and KP SE was differ (Table 1 and 2). The SGR of *C. vulgaris* (TRG 2A) was higher in modified SE of SB SE compared to KP SE. It showed significant differences ($p < 0.05$) between SB and KP SE. *C. vulgaris* (TRG6-B01) showed higher SGR in media + 121 °C twice of KP SE but no significant differences ($p > 0.05$) were observed. Along with SGR, the division rate of *C. vulgaris* (TRG 2A) was higher in SB SE while for *C. vulgaris* (TRG6-B01), the division rate was higher in KP SE. The SGR of microalgae in both SE were increased relative to the control. Nasir et al. (2015) stated that Chlorella sp. growth in Africa catfish (*Clarias gariepinus*) fish farm wastewaters successfully removed NH_3 (98.7 %) and PO_4^3 (92.2 %) producing useful cell mass under non-axenic conditions. Another recent study demonstrated that in the exponential process, the microalgal SGR was (0.16 day^{-1} for *Tetraselmis suecica* and 0.15 day^{-1} for *Dunaliella tertiolecta*) in aquaculture wastewater (Andreotti et al., 2019). In Nile tilapia (*Oreochromis niloticus*) wastewater, *Chlorella sorokiniana* was cultivated heterotrophically where it capable of producing 2.5 g L^{-1} of dry cell mass (Dourou et al., 2020; Guldhe et al., 2017). Thus, the cultivation method or the amount of nutrients decide the various growth values as it has been important factor in the growth of microalgae. The type of aquaculture sludge also influences the microalgae growth. In aquaculture and treated sewage wastewater, *C. vulgaris* and *Scenedesmus obliquus* growth were significantly lower than those acquired in different wastewater containing higher nutrients, for example, urban sewage and pig feedlot wastewater, indicating that the amount of nutrients in aquaculture wastewater is insufficient to support high algal biomass productivity in batch culture mode (Gao et al., 2016).

Table 1. Effects of different modified SE on maximum OD, specific growth rate and division rate of *C. vulgaris* (TRG 2A)

Sludge Extract (SE)	Modified SE	Maximum OD	Specific growth rate, μ	Division rate, k (d^{-1})
	Control	0.30 ± 0.00	0.08 ± 0.00	0.12 ± 0.00
SB SE	Media + 105 °C	0.54 ± 0.00	0.12 ± 0.00	0.17 ± 0.00
	Media + 105 °C twice	0.54 ± 0.00	0.12 ± 0.00	0.18 ± 0.01
	Media + 121 °C	0.55 ± 0.01	0.12 ± 0.00	0.17 ± 0.00
	Media + 121 °C twice	0.59 ± 0.01	0.12 ± 0.01	0.18 ± 0.01
	Media + 24 hours	0.57 ± 0.00	0.13 ± 0.00	0.19 ± 0.01
	Control	0.11 ± 0.02	0.03 ± 0.00	0.04 ± 0.00
KP SE	Media + 105 °C	0.22 ± 0.00	0.07 ± 0.00	0.09 ± 0.00
	Media + 105 °C twice	0.29 ± 0.02	0.08 ± 0.00	0.11 ± 0.00
	Media + 121 °C	0.20 ± 0.04	0.06 ± 0.00	0.09 ± 0.00
	Media + 121 °C twice	0.27 ± 0.01	0.08 ± 0.00	0.11 ± 0.01
	Media + 24 hours	0.14 ± 0.01	0.06 ± 0.00	0.08 ± 0.01

Table 2. Effects of different modified SE on maximum OD, specific growth rate and division rate of *C. vulgaris* (TRG6-B01)

Sludge Extract (SE)	Modified SE	Maximum OD	Specific growth rate, μ	Division rate, k (d^{-1})
	Control	0.30 ± 0.00	0.08 ± 0.00	0.12 ± 0.00
SB SE	Media + 105 °C	0.39 ± 0.01	0.08 ± 0.00	0.12 ± 0.00
	Media + 105 °C twice	0.39 ± 0.00	0.09 ± 0.00	0.13 ± 0.00
	Media + 121 °C	0.42 ± 0.00	0.10 ± 0.00	0.14 ± 0.00
	Media + 121 °C twice	0.42 ± 0.03	0.10 ± 0.00	0.15 ± 0.00
	Media + 24 hours	0.38 ± 0.01	0.09 ± 0.00	0.13 ± 0.01
	Control	0.24 ± 0.03	0.06 ± 0.00	0.09 ± 0.01
KP SE	Media + 105 °C	0.34 ± 0.04	0.08 ± 0.00	0.12 ± 0.01
	Media + 105 °C twice	0.45 ± 0.01	0.10 ± 0.00	0.15 ± 0.00
	Media + 121 °C	0.46 ± 0.00	0.11 ± 0.00	0.16 ± 0.00
	Media + 121 °C twice	0.34 ± 0.00	0.12 ± 0.00	0.17 ± 0.00
	Media + 24 hours	0.24 ± 0.03	0.06 ± 0.00	0.09 ± 0.00

4. Conclusion

In conclusion, the study showed the possibility of enhanced microalgae growth with additional enrichment from treated sludge extracts. Autoclaving sludge for extended time at high temperatures could increase the microalgae growth. The microalgae growth depends on the sludge type used. The consistency of the SE and the type of microalgae examined decides the result of any enrichment studies and the possible future use of the mass cultivation.

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