Malaysian Journal of Microbiology, Vol 18(5) 2022, pp. 463-472
DOI: http://dx.doi.org/10.21161/mjm.211333

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Enhanced microbial biomass and lipid production through co-cultivation of yeast *Rhodotorula toruloides* and microalga *Chaetoceros muelleri*

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Received 25 October 2021; Received in revised form 25 May 2022; Accepted 28 June 2022

ABSTRACT

Aims: The synergistic bio-activity between oleaginous yeast and microalga has been recognized, which would enhance lipid production as biodiesel feedstock. Nevertheless, yeast and microalga require different conditions for optimal growth. In this study, the locally isolated oleaginous yeast *Rhodotorula toruloides* and microalga *Chaetoceros muelleri* were co-cultivated to enhance biomass and lipid production.
Methodology and results: The growth characteristics of both yeast and microalga monocultures were initially determined prior to optimizing the co-cultivation conditions. The biomass and lipid productivity of the co-culture were investigated and compared to their monocultures. The results showed that *R. toruloides* grew actively within 3 days while *C. muelleri* exhibited more prolonged cultivation, up to 21 days. The co-cultivation could be carried out optimally using growth media at pH 6, light intensity of 15,000 lux and yeast/microalga ratio of 1:2, yielding the highest biomass productivity determined at 0.18 g/l/day and lipid production of 17%. The lipid productivity of the co-culture increased by 42% and 75% as compared to monocultures of yeast and microalga, respectively. Furthermore, the biomass productivity was also higher than the monoculture, about 1.2-fold for the yeast and 13-fold for the microalga.
Conclusion, significance and impact of study: The findings revealed that co-cultivation of yeast and microalga is a viable technique for long-term microbial oil production.

Keywords: Lipid production, *Rhodotorula toruloides*, *Chaetoceros muelleri*, co-cultivation, oleaginous microorganisms

INTRODUCTION

The growing worldwide population has led to the increasing bioenergy demand as a substitute for depleting fossil fuels. Petroleum-based fuels play a significant role in daily human life; however, they are non-renewable and known for their negative environmental impacts. Excessive usage of coal, natural gas and petroleum has resulted in severe global warming and greenhouse gas emissions (Peng *et al.*, 2020). Renewable energy has lessened the detrimental effects of hydrocarbon oils (Haldar and Sethi, 2022). For the last two decades, the advent of biodiesel as a promising alternative to fossil fuel has attracted increasing attention and support. Biodiesel is regarded as environmentally friendly, with a low combustion profile and minimal release of harmful substances to the environment (Singh *et al.*, 2020). Biodiesel with determined properties such as viscosity, density and melting point can be blended in any ratio with the standard diesel fuel and can be used in diesel engines without modification (Ogunkunle and Ahmed, 2019).

Various sources have been utilized as feedstock for the production of biodiesel, including plant oils, animal fats and waste cooking oils (Anwar, 2021). Plant feedstocks such as rapeseed and soybean oil have been extensively used for biodiesel production. Nevertheless, plant oils are under elevating pressure due to the demand for natural resources and arable land (Singh and Dhar, 2011). Biofuel production from plant feedstocks competes for food resources; hence, a less ideal substitute for finite fossil fuel resources. Recently, there has been a focus on advancing microbial oils as a source of energy. Many microbes, including bacteria, yeast, fungus and algae, have been discovered to store lipids (Qadeer *et al.*, 2017). Oleaginous bacteria accumulate considerable amounts of lipids known as single-cell oils under nutrient-limiting conditions with excess carbon. These lipids are mostly made up of neutral triacylglycerols (TAGs), which are energy-dense fatty acids that can be used to make biodiesel (Patel *et al.*, 2019). Microbial oils are gaining popularity due to their short life cycle and simplicity of scale-up, as well as their lack of sensitivity to location, season or environment. Furthermore, when low-cost

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

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carbon sources such as palm oil mill effluent (Ahmad *et al.*, 2019) and textile wastewater effluent are used as growth media (Wu *et al.*, 2017), the lipid generated not only has similar fatty acid compositions to plant and animal oils, but it is also more cost-effective.

Yeasts can produce a large quantity of lipid content using a variety of carbon sources, including glycerol and molasses (Yen *et al.*, 2015). Oleaginous yeasts have been reported to be capable of synthesizing and accumulating high amounts of intracellular triglycerides (TAG) that can account for up to 70% of their biomass weight (Bansal *et al.*, 2020). Beopoulos and Nicaud (2012), in their study, reported that lipid content and lipid profile differed amongst species. Among the lipid-accumulating oleaginous yeasts identified were *Cryptococcus albidus*, *Lipomyces lipofera*, *L. starkeyi*, *Rhodosporidium toruloides*, *Rhodotorula glutinis*, *Trichosporon pullulan* and *Yarrowia lipolytica*. *Rhodotorula glutinis* has been observed producing a large amount of lipid, up to 49%, with 61% comprising of oleic acid content (Maza *et al.*, 2020).

On the other hand, oleaginous microalga has also been identified as an effective biodiesel feedstock due to its high lipid content, photosynthesis capacity and carbon dioxide reduction efficiency. According to Moshood *et al.* (2021), microalga species with higher lipid content material can be utilized as feedstock for jet fuel, biodiesel and biogasoline. Microalga can convert carbon dioxide into lipids through photosynthesis, making them known as cell factories of sunlight-driven. Some microalgal cells can develop in a heterotrophic or mixotrophic system instead of a strict photosynthesis system due to the availability of organic and inorganic substrates (Eugenia, 2012). Algal culture is well-known for its ability to fix carbon (Kumar *et al.*, 2011), which is then absorbed into carbohydrates and lipids, allowing it to create chemicals, energy or food (Dani *et al.*, 2021).

Co-cultivation of oleaginous microorganisms has been shown to provide synergistic benefits over single organisms in lipid production. Yeasts and microalga could cooperate symbiotically via gas exchanges that are advantageous for biomass growth (Berthold *et al.*, 2019). The synergistic activity may stimulate high biomass production by the co-culture. A higher biomass concentration has been highly pursued in microbial oil technology as the accumulation of lipid typically occurs in the biomass cultures during nutrient-depleted conditions (Berthold *et al.*, 2019). In the presence of light, microalga would act as an oxygen generator for yeast, while yeast would deliver carbon dioxide to microalga via aerobic respiration. As a result of the symbiotic activity, the metabolites and lipids production could be increased (Cheirsilp *et al.*, 2011).

However, attempts to co-cultivating yeast and microalga are often challenged by their different growth requirements, such as optimal pH condition, temperature, light necessity and shear sensitivity. For instance, media preference for both yeast and microalga might be different. In addition, the cultivation of microalga is very sensitive to light intensity (Bhuyar *et al.*, 2021). While agitation is beneficial for greater yeast productivity, microalga has been recognized as a shear-sensitive culture whereby agitation causes cells destruction (Michels *et al.*, 2016). Yeast, on the other hand, has been found to overwhelm the growth of microalga, as demonstrated in a few studies involving the screening of alga-yeast co-culture (Liu *et al.*, 2018). *Rhodotorula glutinis* yeast was reported to dominate the cell growth when co-cultivated with *Chlorella vulgaris* microalga in industrial wastewater (Zhang *et al.*, 2014). Although the co-cultivation of microorganisms has been widely studied, the research of microalga-yeast mixed cultures is considerably still in its early stages due to the wide variety of the co-culture possible combinations. Therefore, the search for a mutual optimal co-cultivation of yeast and microalga has been a biotechnological interest, hence the objective of this study.

Yeast *Rhodotorula toruloides* and microalga *Chaetoceros muelleri* were co-cultivated in this study for improved biomass and lipid production. Their individual growth profile was initially examined and subsequently, the optimal conditions for co-cultivation were investigated. The findings of this study provide insightful knowledge on the utilization of local oleaginous microorganisms for sustainable and industry-driven fuel production.

MATERIALS AND METHODS

Culture media and growth conditions for yeast

Yeast, *R. toruloides* was isolated from a runoff water stream at a copper mine site located in Sabah, Malaysia. The strain species have been identified in a previous study (Geoffrey, 2018). Yeast was grown on a sterilized Yeast Extract-Peptone-Dextrose (YPD) agar plate containing bacteriological peptone (20 g/L), yeast extract (10 g/L), glucose (20 g/L) and agar (20 g/L) and incubated at 30 °C (Kot *et al.*, 2019). Media sterilization was conducted at 121 °C for 15 min. For inoculum preparation, a few loops of overnight-grown *R. toruloides* colonies were inoculated into YPD broth containing peptone (20 g/L), yeast extract (10 g/L) and glucose (20 g/L). The yeast culture was incubated at 30 °C with 200 rpm agitation. Estimation of inoculum cell concentration was carried out by measurement of optical density of the yeast culture at 600 nm wavelength. Inoculum at mid-log phase as determined by the optical density measurement using spectrophotometer within range of OD_{600nm} 2.0-2.2 was collected for yeast cultivation for lipid production.

Culture media and growth conditions of microalga

Microalga *C. muelleri* was taken from the culture collection of the Unit for Harmful Algal Blooms Studies (UHABs) of Borneo Marine Research Institute, Universiti Malaysia Sabah. The microalga *C. muelleri* was isolated from a mangrove swamp of Kota Kinabalu Wetland using a plankton net of 20 µm mesh size. The algal cells were cultured and maintained in Walne's Medium under normalized conditions at 27 °C, 16:8 h of light:dark cycle

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(5) 2022, pp. 463-472
DOI: http://dx.doi.org/10.21161/mjm.211333

under illuminance of 5,000 lux (70 µmol/m^2/s) (Chin, 2017). The Walne's media preparation was according to Walne (1970). The Walne's medium was prepared by adding a series of stock solutions after sterilization at 121 °C for 15 min. The algal culture was placed inside a Versatile Environmental Test Chamber MLR-325H (Panasonic, Japan) and incubated at 27 °C. The growing cultures were harvested after 10 days of incubation or OD_{680nm} 0.7-0.9 for inoculum preparation. The measurement of optical density of the algal culture was spectrophotometrically determined at 680 nm wavelength.

Determination of growth characteristics

The growth profile of the *R. toruloides* and *C. muelleri* was observed from the day of inoculation until the cells entered their death phase. The inoculation was conducted using 10% (v/v) of yeast or algal cultures into 250 mL flasks containing 150 mL of YPD broth and Walne's media, respectively. Culture monitoring was conducted by measuring the optical density of the culture at a wavelength of 600 nm for *R. toruloides* and 680 nm for *C. muelleri.* The growth culture of yeast was monitored every 6-h interval, while the growing culture of microalga was observed every 3-day interval. The optical density of culture during the exponential phase at different pH conditions for each culture was monitored. The physical appearances of the yeast and microalga cultures were also observed.

Optimization of yeast and microalga co-cultivation

Co-cultivation of *R. toruloides* and *C. muelleri* cultures was carried out using the method described by Yen *et al.* (2015) with slight modification. The operating conditions for the co-cultivation were optimized, including pH, yeast/microalga ratio and light intensity. The pH conditions were varied within the range of pH 5-8, while the yeast/microalga ratio and light intensity were fixed at 1:1 and 15000 lux, respectively. Next, the yeast to microalga ratio (1:1, 1:2, 1:3, 1:4) was optimized using initial pH media set at 7 and light intensity at 15000 lux. Finally, optimal light intensity was studied (5000, 15000, 22000) in experiments using pH media at 7 and 1:1 yeast to microalga ratio. A total of 10% (v/v) inoculum (15 mL) containing yeast and microalga cultures at different ratios (1:1, 1:2, 1:3, 1:4) were cultured in sterilized media of YPD broth (67.5 mL) and Walne's media (67.5 mL). For the 1:1 ratio, a total of 7.5 mL of log-phase *R. toruloides* (OD_{600nm} 2.0-2.2) and 7.5 mL *C. muelleri* (OD_{680nm} 0.7-0.9) were added to the YPD-Walne's growth media. The media was adjusted at varying initial pH (5-8). The light intensity of the environmental chamber was varied at 5000, 15000 and 22000 lux and was set to 16:8 light and dark cycle. The cultivation condition was maintained under normalized conditions at 27 °C inside the environmental chamber. Samples were taken for biomass and lipid production analyses after 10 days of incubation. The co-cultivation study was further conducted using the optimal conditions and compared with the yeast and microalga monocultures up to 40 days of cultivation.

Determination of biomass production

Samples were spun down at 3400× *g* for 5 min and washed with deionized distilled water. After that, the samples were frozen overnight at -80 °C temperature. Next, the samples were freeze-dried using FreeZone Freeze Dry System (LABCONCO, USA) for 2 days. The freeze-dried samples were crushed into fine powder. The gravimetrically measured biomass was used to determine the biomass concentration (g/l/day) using Equation (1).

$$\text{Biomass productivity} = (x_2 - x_1)/(t_2 - t_1) \quad \text{(1)}$$

Where x_2 and x_1 are the biomass dry weight concentration on day t_1 (start point of cultivation) and t_2 (endpoint of cultivation), respectively.

Determination of lipid production

The lipid extraction was carried out according to the method described by Bligh and Dyer (1959) with slight modifications. The freeze-dried biomass samples were ground into powder and deposited in a glass tube containing around 3-5 mg samples. Methanol, chloroform and water in a 1:1:1 ratio were added to the sample. The solution mixes were vortexed to homogenize. The mixture was left to settle for an hour at room temperature. After centrifuging the solution in a speed-vac for about 30 min, complete separation was obtained. Two visible layers were obtained: the polar zone at the upper layer, which consisted of methanol and water and the non-polar at the lower region, which consisted of chloroform and lipid. The top layer was discarded, while the bottom layer was transferred to a pre-weighed glass tube and dried for 1.5 h in a speed-vac at 30 °C. The glass tube containing the crude lipid was weighed after drying. The extracted lipid (g), lipid content (%) and lipid productivity (g/l/day) were calculated using Equations (2-4), respectively.

Lipid (g)
= (Weight of glass tube + Extracted oil) - (Weight of glass tube) …….……... (2)

Lipid Content (%)
= [Extracted lipid (g)/Sample weight (g)] × 100 ….…… (3)

Lipid Productivity (g/l/day)
= Lipid content × Biomass Productivity ……..…… (4)

Statistical analysis

Sample collection and analysis were carried out from triplicate independent experiments. The data were analyzed using the statistical tool in MS Excel 2019. Each value corresponds to the mean ± standard deviation of three biological samples.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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DOI: http://dx.doi.org/10.21161/mjm.211333

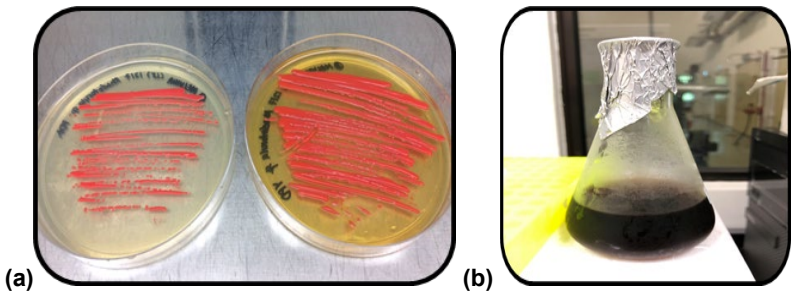

Figure 1: (a) The colonies and cultures of yeast *R. toruloides* grown in YPD agar plate and YPD broth media, respectively, for three days.

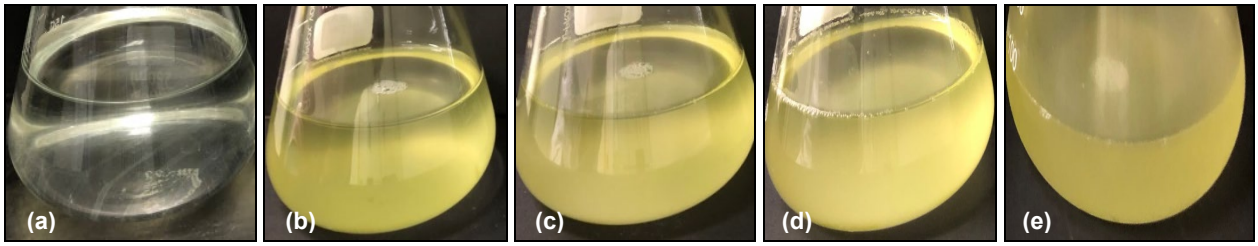

Figure 2: Microalga *C. muelleri* grown in Walne's broth media at (a) day 0, (b) day 10, (c) day 20, (d) day 30 and (e) day 40.

RESULTS AND DISCUSSION

Physical observation of *R. toruloides* and *C. muelleri*

Microbial proliferation is the most vital response of microorganisms to their physiochemical environment. When a seed culture is inoculated into a nutrient media, the organisms actively proliferate by taking up and converting dissolved nutrients into biomass (Shuler *et al.*, 2017). Figure 1 shows the physical appearance of yeast *R. toruloides* after 3 days of cultivation on YPD agar plate and YPD broth. The culture appeared red on YPD agar media as shown in Figure 1a and was observed dark brown in broth media (Figure 1b). The culture turned red on the agar media (Figure 1a) and appeared dark brown in broth media (Figure 1b). According to Saenge *et al.* (2011), *R. toruloides* colonies are red in color due to the presence of carotenoid compounds. *R. toruloides* can produce high lipid when grown in nitrogen-limiting media and also can metabolize other economically relevant feedstocks, including acetic acid, glycerol and inulin (Sambles *et al.*, 2017).

Meanwhile, the colorless Walne's media (Figure 2a) turned into a greenish-yellow color after the cultivation of *C. muelleri* (Figure 2b-e). As the culture progressed from day 20 (Figure 2c) to day 40 (Figure 2e), de-greening of the color may have occurred due to chlorophyll biodegradation during microalga cultivation as described by Hörtensteiner *et al.* (2000). This was in line with a previously published study which found that cells' chloroplasts were degraded after 48 h of heterotrophic metabolism, resulting in the loss of the cells' thylakoid membranes as a phenomenal seen in Figure 2e (Xiong *et al.*, 2010). In much earlier research, *C. muelleri* was found to exhibit a rapid growth rate and tolerance over a broad range of specific conductances and temperatures and yielded large quantities of neutral lipid (McGinnis *et al.*, 1997).

Growth characteristic of *R. toruloides* and *C. muelleri*

Figure 3 shows the growth profile of *R. toruloides* and optical density in a variety of media pH conditions. Both cultures demonstrated a typical growth curve of yeast with a lag phase, exponential phase, stationary and death phase. The yeast took about 13 to 14 h of stagnant growth, indicating a lag phase of yeast adapting to a new environment (Figure 3a). The yeast entered an exponential phase afterward and multiplied rapidly from 15 h to 30 h cultivation. From 30 to 50 h of incubation, the cells' growth slowed, indicating that they were in the stationary phase. After 60 h of cultivation, the cells finally reached their death phase. During the yeast cultivation, a cloudy broth was observed, which was most likely due to the presence of dense biomass, metabolites and toxic products as a result of the cells' growth (Mackay *et al.*, 2015). As the pH level increased, the yeast showed an increasing trend of growth. The findings showed greater optical density was obtained when using pH 6 to 7. The finding is in agreement with Cheirsilp *et al.* (2012) reported that *R. toruloides* grew best in slightly acidic conditions (pH 6). The yeast strain was discovered in copper mine soil containing metals such as iron, aluminium and copper (Low *et al.*, 2020). The site's seepage water was discovered in an acidic environment due to the high metal content of the soil. The yeast may

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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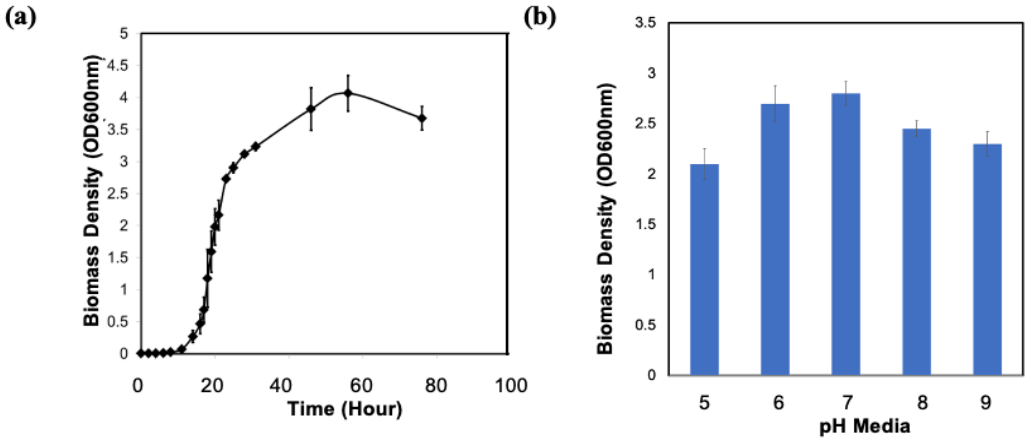

Figure 3: (a) Growth profile of *R. toruloides* and (b) the optical density at mid-log phase culture at different pH of media in YPD medium. Triplicate biological samples were prepared and data are shown as mean ± SD.

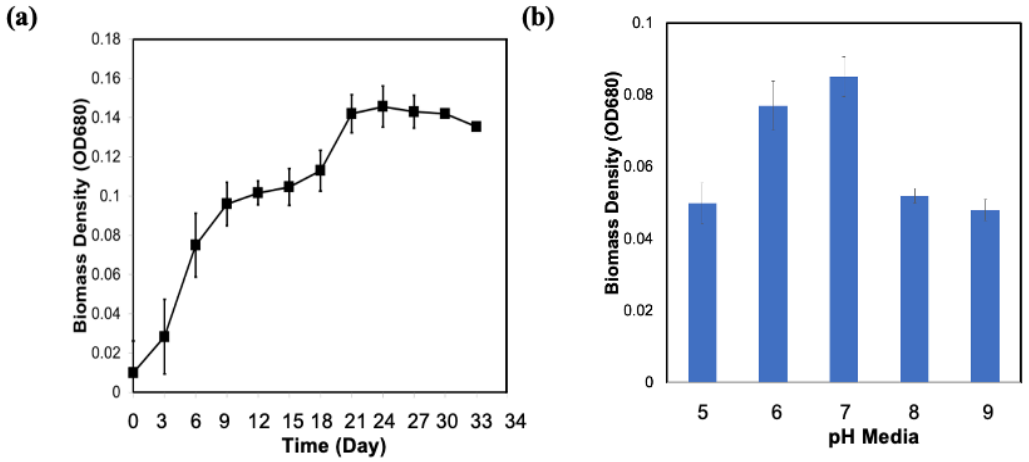

Figure 4: (a) Growth profile of *C. muelleri* and (b) the optical density at mid-log phase culture at different pH of media in YPD medium. Triplicate biological samples were prepared, and data are shown as mean ± SD.

have been acclimatized under such extreme conditions, therefore, explaining the remarkable biomass production around pH 6 to pH 7 in comparison to the alkaline state.

Figure 4 shows the growth profile and biomass output of the microalga *C. muelleri*. The microalga grew rapidly until day 12 with no discernible lag period at the beginning of cultivation (Figure 4a). A slower rate of exponential phase was observed afterward before reaching the stationary phase on day 21. According to Shuler *et al.* (2017), although there is no increase in growth during the stationary phase due to the same rate of cell growth and death, cells are still metabolically active and produce secondary metabolites. After 33 days, *C. muelleri* exhibited a decreasing trend of growth, indicating the commencement of the death phase. Similar to *R. toruloides*, *C. muelleri* proliferated best under a neutral condition (pH 7) with an optical density (OD_{680}) of approximately 0.09 (Figure 4b). The growth of the microalga was suppressed at stronger alkaline (pH 8-9) and acidic (pH 5) media. According to Hansen (2002), pH condition affects the carbon dioxide distribution and availability of nutrient supply that promotes the growth of microalgae. On the other hand, when the pH level dropped to more acidic conditions, the nutrient uptake could be affected, resulting in the inhibition of the microalgal growth as described by Juneja *et al.* (2013).

The findings of this study reveal a mutual pH preference around pH 6 to 7 for both cultures. The *R. toruloides* was found a fast grower culture than *C. muelleri*. Both *R. toruloides* and *C. muelleri* were further co-cultivated to increase lipid production through their symbiotic bio-activities. The inoculum for co-cultivation was prepared using active seed cultures obtained in the middle of their log phase. Mid-log phase for *R. toruloides* was discovered around 20 h of cultivation with cell density ranging from 1.8 to 2.2. (OD_{600}). Meanwhile, the mid-log phase for *C. muelleri* occurred at 9 to 10 days of cultivation, with cell density determined within 0.06 to 0.08 (OD_{680}). Both cultures were further co-cultivated and optimized to enhance biomass and lipid production.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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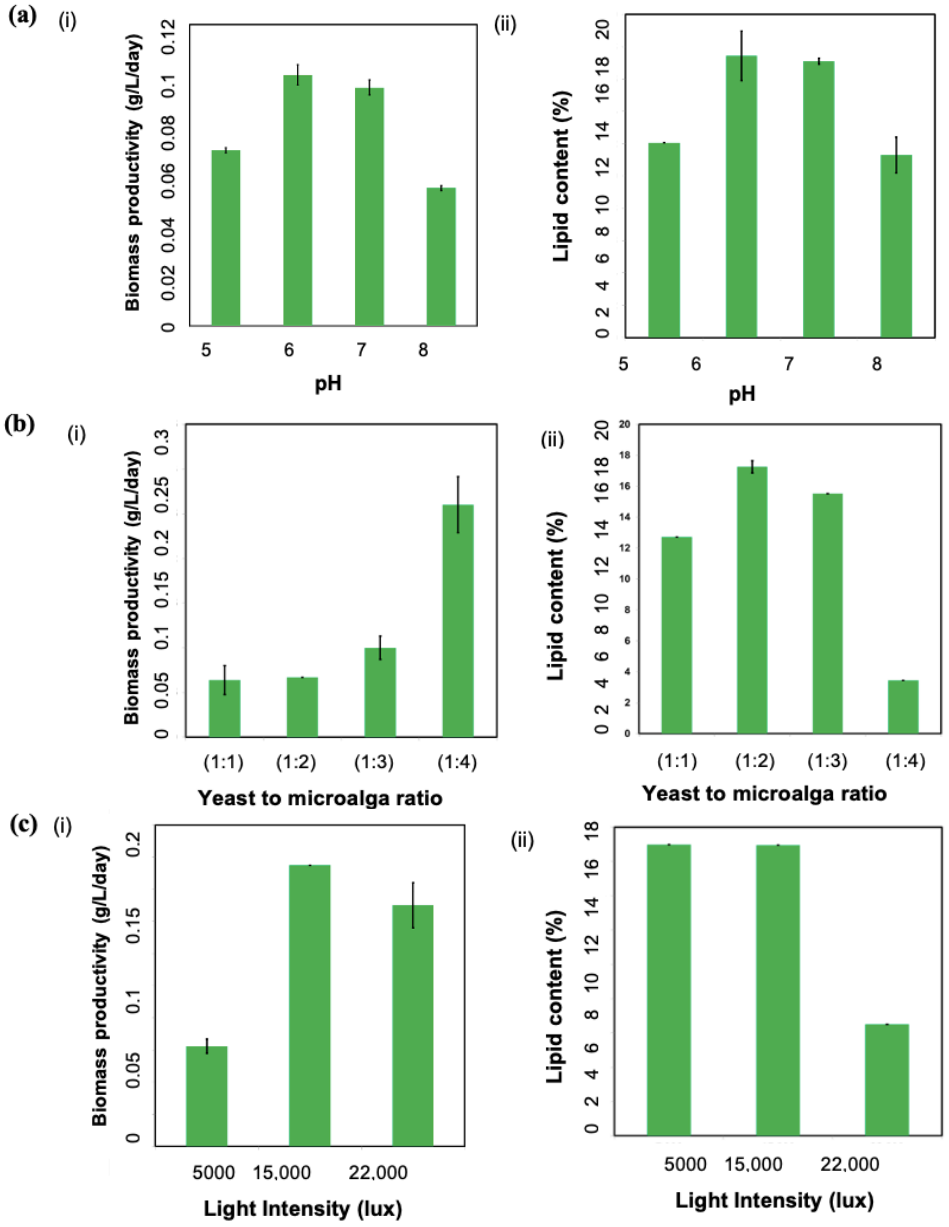

Figure 5: Effect of (a) pH, (b) yeast to microalga ratio and (c) light intensity on (i) biomass productivity and (ii) lipid content.

Optimization of *R. toruloides* and *C. muelleri* co-cultivation

The *R. toruloides* and *C. muelleri* co-cultivation conditions were investigated compared to that of a monoculture. The effect of initial pH of cultivation media, light intensity and inoculum ratio of yeast and microalga on lipid production were investigated as presented in Figure 5. Figure 5a presents the effect of pH media on biomass productivity [Figure 5 a(i)] and lipid content [Figure 5 a(ii)] of the co-culture. As can be seen, the amount of lipid corresponded to the amount of biomass. The highest biomass productivity of nearly 0.1 g/l/day and lipid content of around 18% was obtained at pH 6 and 7. Highly acidic and alkaline states of media were found unfavorable for the co-cultures and significantly reduced biomass and lipid production. Some drops in pH media levels were observed in common yeast cultivation, most likely due to the production of organic acids. When the media broth was acidified by the organic acids co-products, yeast growth was reported to be inhibited, as reported by Cheirsilp *et al.* (2011). Interestingly, Xue *et al.* (2010) discovered that metabolites produced by yeasts, such as pyruvic and acetic acids, may be advantageous to the

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growth of microalga in a co-culture environment. Less pyruvic acid was detected in their yeast-microalga co-cultivation, which was presumed to be utilized by the microalga *S. platensis*, while the acetate was utilized by the culture as a carbon source during the mixotrophic process. According to Zhao *et al.* (2012), when the culture contains both inorganic and organic substrates, certain algal cells can thrive not only in a photosynthetic system but also in a heterotrophic system or mixotrophy. As a result, organic acid-induced suppression of yeast growth might be avoided in yeast-microalga co-cultivation.

The inoculation condition is an essential requirement to enhance bio-production efficiency (Padmaperuma *et al.*, 2018). The optimal inoculum density was determined by inoculating media with varying yeast/microalga ratios ranging from 1:1 to 1:4. The biomass production increased in corresponding to the microalgal concentration used as inoculum (Figure 5b). The yeast/microalga ratio of 1:4 yielded the highest biomass productivity determined at 0.25 g/l/day [Figure 5b(i)]. Nonetheless, the 1:4 ratio produced the least amount of lipid, which was 4.5-fold lesser than the 1:2 ratio that yielded the most amount of lipid (17.3%), as Figure 5b(ii) indicates. The enhancement of lipid production at a 1:2 ratio could be due to an improved symbiosis relationship between the yeast and microalga under the optimized seed ratio.

Cheirsilp and Torpee (2012) described the seed ratio, along with the carbon dioxide uptake and culturing method, could affect the growth of both autotrophs and heterotrophs. Moreover, this optimal ratio may contribute to the media condition under nitrogen-limiting conditions, leading to the formation of lipid. It has been reported that lipids and triglycerides tend to accumulate more in the cells of yeast and microalga under the condition of nitrogen limitation with an excess of carbon compounds (Fakhry and El-Maghraby, 2015; Wang *et al.*, 2018). A decrease in lipid productivity at the highest biomass of seed culture also has been seen in the study by Zhang *et al.* (2014) during co-cultivation of yeast *R. glutinis* with microalga *C. vulgaris*.

The light requirement is another essential factor to be addressed associated with microalgal growth. Light serves as a source of energy for the conversion of carbon dioxide into organic molecules. In this work, the influence of light illumination on co-culture was studied at varying light intensities (5,000 lux, 15,000 lux, 22,000 lux) using 16:8 light/dark photoperiods (Figure 5c). The initial pH media was adjusted at 7.0 and a cool white fluorescent light was employed. It was found that higher light intensity was affirmative for the growth of microalga to carry out photosynthesis reaction. The outcome seen here also corroborates the findings of previous studies. Low light level causes growth limitations, as described by Carvalho *et al.* (2010). Meanwhile, Su *et al.* (2012) found that continuous lighting enhanced biomass concentration and dissolved oxygen in *Chlamydomonas reinhardtii*, *Scenedesmus rubescens* and *Chlorella vulgaris* for nutrient removal on wastewater when compared to altered illumination and non-illuminated culture. The major drop in lipid production at 22,000 light intensity [Figure 5c(ii)] is most likely due to an excess of light intensity supply which reduced the biomass productivity, as seen in Figure 5c(i). The light should be provided at the appropriate intensity, duration, and wavelength for optimal growth and productivity of microalga. Excessive intensity may lead to photooxidation and growth inhibition (Carvalho *et al.*, 2010).

To summarise, the optimal co-cultivation condition for *R. toruloides* and *C. muelleri* were achieved at pH 6 of media, yeast, and microalga ratio at 1:2 and a light intensity of 15,000 lux. The optimum conditions were further evaluated for biomass and lipid production in comparison with their respective individual culture.

Comparison of co-culture and monoculture

The biomass and lipid productivity of mono and co-culture *R. toruloides* and *C. muelleri* are presented in Figure 6. Longer cultivation, up to 40 days, was employed to observe the trend of biomass and lipid productivity to ensure nitrogen-limiting condition was reached for a greater lipid accumulation. In addition, the microalga was found a slow grower culture, as observed in Figure 2, reaching up to 33 days to still remain in the stationary phase. As can be seen, the highest biomass productivity was obtained by the monoculture of *R. toruloides* about 0.5 g/l/day for a 10-days cultivation period (Figure 6a). A prolonged cultivation time has resulted in a rapid reduction of the biomass productivity to 0.1 g/l/day at 40 days of cultivation. *C. muelleri* produced the least amount of biomass (below 0.1 g/l/day) throughout the cultivation period. Meanwhile, the co-culture of *R. toruloides* and *C. muelleri* yielded the highest biomass productivity (0.23 g/l/day) at 20 days cultivation period. The yield was 1.25-fold and 5-fold higher than the monoculture of *R. toruloides* and *C. muelleri*, respectively, up to 20 days of cultivation. It was observed that the biomass production of the co-culture was higher than the monoculture till the end of cultivation time, signifying the benefit of synergistic activity of the co-culture. The yeast-microalga synergistic activity in terms of gas exchange described by Dias *et al.* (2019) may have contributed to the accumulation of biomass in comparison to monoculture.

The production of lipid, on the other hand, was inversely proportional to the incubation time mainly for the co-culture and *C. muelleri* monoculture (Figure 6b). As the incubation duration was increased from 10 to 40 days, a declining trend in lipid production was observed. The monoculture of *R. toruloides* also exhibited a notable decrease in lipid productivity after 30 days of cultivation. Interestingly, similar to biomass productivity, optimal lipid production was also observed within 20 days of cultivation. The lipid productivity at 10 days of cultivation was greater than the monoculture of *C. muelleri* (4 times) and *R. toruloides* (3.2 times). An enhancement in lipid synthesis by up to 12% was also observed in a mixed culture of *Rhodosporidium toruloides* and *Chlorella vulgaris* (Zeng *et al.*, 2018). A higher biomass and lipid productivity were observed for the monoculture of *R.*

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(5) 2022, pp. 463-472
DOI: http://dx.doi.org/10.21161/mjm.211333

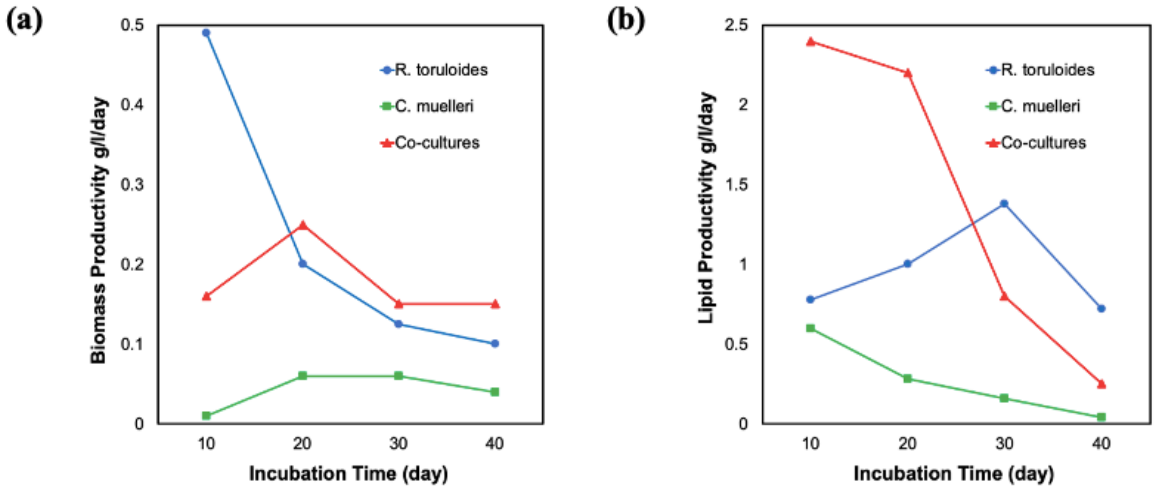

Figure 6: Biomass productivity of monoculture and co-culture of yeast *R. toruloides* and microalga *C. muelleri* cultivated at pH 6 of media, yeast and microalga ratio at 1:2, and a light intensity of 15,000 lux.

toruloides than *C. muelleri*. It is probably because of *R. toruloides* is a fast grower cell and might yield higher lipid productivity than microalga cultivation.

Overall, the biomass productivity and lipid productivity was dominated by the co-culture, which might be attributed to the synergistic bio-activities of the cultures. The results collectively demonstrated the suitability of yeast-microalga co-culturing for enhanced biomass and lipid production. Higher yield and productivity were dominated by the co-culture which might be attributed to their synergistic bio-activities during cultivation.

CONCLUSION

Yeast *R. toruloides* was found to be a fast grower than microalga *C. muelleri* took up to 21 days to reach the stationary phase. Both monocultures demonstrated a mutual preference toward neutral pH and slightly acidic media for growth. Media at pH 6.0, yeast/microalga ratio of 1:2 and light intensity at 15,000 lux have been identified as the critical determinant for optimal *R. toruloides* and *C. muelleri* co-cultivation. The co-cultivation of the cultures was found to successfully enhance the biomass and lipid productivity for 20 days of cultivation compared to their monoculture counterparts. The findings of this study provide key knowledge for the development of new technology for enhancing microbial oil production. Further advancement in this field can be considered by cultivating the co-culture in low-cost media such as agricultural waste effluents in the future for simultaneous green waste management and economical biofuel production.

ACKNOWLEDGEMENTS

University Malaysia Sabah funded the project under the Niche Grant Scheme (SDN0024-2019). Special thanks to Assoc. Prof. Dr. Kenneth Francis Rodrigues and the research team for providing valuable research samples.

REFERENCES

Ahmad, F. B., Zhang, Z., Doherty, W. O. S. and O'Hara, I. M. (2019). The prospect of microbial oil production and applications from oil palm biomass. *Biochemical Engineering Journal* **143, 9-23.**

Anwar, M. (2021). Biodiesel feedstocks selection strategies based on economic, technical, and sustainable aspects. *Fuel* **283, 119204.**

Bansal, N., Dasgupta, D., Hazra, S., Bhaskar, T., Ray, A. and Ghosh, D. (2020). Effect of utilization of crude glycerol as substrate on fatty acid composition of an oleaginous yeast *Rhodotorula mucilagenosa* IIPL32: Assessment of nutritional indices. *Bioresource Technology* **309, 123330.**

Beopoulos, A. and Nicaud, J. M. (2012). Yeast: A new oil producer? *Oléagineux, Corps Gras, Lipides* **19(1), 22-28.**

Berthold, D. E., Shetty, K. G., Jayachandran, K., Laughinghouse, H. D. and Gantar, M. (2019). Enhancing algal biomass and lipid production through bacterial co-culture. *Biomass and Bioenergy* **122, 280-289.**

Bhuyar, P., Sundararaju, S., Rahim, M. H. A., Ramaraj, R., Maniam, G. P. and Govindan, N. (2021). Microalgae cultivation using palm oil mill effluent as growth medium for lipid production with the effect of CO_2 supply and light intensity. *Biomass Conversion and Biorefinery* **11, 1555-1563.**

Bligh, E. G. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37(8), 911-917.**

Carvalho, A. P., Silva, S. O., Baptista, J. M. and Malcata, F. X. (2010). Light requirements in

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

microalgal photobioreactors: An overview of biophotonic aspects. *Applied Microbiology and Biotechnology* **89, 1275-1288.**

Cheirsilp, B. and Torpee, S. (2012). Enhanced growth and lipid production of microalgae under mixotrophic culture condition: Effect of light intensity, glucose concentration and fed-batch cultivation. *Bioresource Technology* **110, 510-516.**

Cheirsilp, B., Kitcha, S. and Torpee, S. (2012). Co-culture of an oleaginous yeast *Rhodotorula glutinis* and a microalga *Chlorella vulgaris* for biomass and lipid production using pure and crude glycerol as a sole carbon source. *Annals of Microbiology* **62, 987-993.**

Cheirsilp, B., Suwannarat, W. and Niyomdecha, R. (2011). Mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* for lipid production from industrial wastes and its use as biodiesel feedstock. *New Biotechnology* **28(4), 362-368.**

Chin, G. (2017). Microalgal culture techniques. *In*: Techniques in Molecular Biology. Rodrigues, K. (ed.). Biotechnology Research Institute, Sabah, Malaysia.

Dani, N., Zare, D., Assadi, M. M., Irani, S. and Soltani, N. (2021). Isolation, screening and medium optimization of native microalgae for lipid production using nutritional starvation strategy and statistical design. *International Journal of Environmental Science and Technology* **18(10), 2997-3012.**

Dias, C., Santos, J., Reis, A. and da Silva, T. L. (2019). Yeast and microalgal symbiotic cultures using low-cost substrates for lipid production. *Bioresource Technology Reports* **7, 100261.**

Eugenia, J. O. (2012). Dual purpose microalgae-bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a biorefinery. *Biotechnology Advances* **30(5), 1031-1046.**

Fakhry, E. M. and El-Maghraby, D. M. (2015). Lipid accumulation in response to nitrogen limitation and variation of temperature in *Nannochloropsis salina*. *Botanical Studies* **56, 6.**

Geoffrey, I. (2018). Optimization of lipid production for biodiesel feedstock by *Rhodotorula* sp. isolated from Mamut Copper Mine, Sabah. M.Sc. Thesis. Universiti Malaysia Sabah, Malaysia.

Haldar, A. and Sethi, N. (2022). Environmental effects of Information and Communication Technology - Exploring the roles of renewable energy, innovation, trade and financial development. *Renewable and Sustainable Energy Reviews* **153, 111754.**

Hansen, P. J. (2002). Effect of high pH on the growth and survival of marine phytoplankton: Implications for species succession. *Aquatic Microbial Ecology* **28(3), 279-288.**

Hörtensteiner, S., Rodoni, S., Schellenberg, M., Vicentini, F., Nandi, O. I., Qui, Y. L. and Matile, P. (2000). Evolution of chlorophyll degradation: The significance of RCC reductase. *Plant Biology* **2(1), 63-67.**

Juneja, A., Ceballos, R. M. and Murthy, G. S. (2013). Effects of environmental factors and nutrient availability on the biochemical composition of algae for biofuels production: A review. *Energies* **6(9), 4607-4638.**

Kot, A. M., Błażejak, S., Kieliszek, M., Gientka, I. and Bryś, J. (2019). Simultaneous production of lipids and carotenoids by the red yeast *Rhodotorula* from waste glycerol fraction and potato wastewater. *Applied Biochemistry and Biotechnology* **189, 589-607.**

Kumar, K., Dasgupta, C. N., Nayak, B., Lindblad, P. and Das, D. (2011). Development of suitable photobioreactors for CO_2 sequestration addressing global warming using green algae and cyanobacteria. *Bioresource Technology* **102(8), 4945-4953.**

Liu, L., Chen, J., Lim, P. E. and Wei, D. (2018). Dual-species cultivation of microalgae and yeast for enhanced biomass and microbial lipid production. *Journal of Applied Phycology* **30(6), 2997-3007.**

Low, Y. Y., Chin, G. J. W. L., Joseph, C. G., Musta, B. and Rodrigues, K. F. (2020). Bacterial diversity of the abandoned mamut copper mine in Sabah, Malaysia and its correlation with copper contamination. *Malaysian Journal of Microbiology* **16(5), 414-424.**

Mackay, S., Gomes, E., Holliger, C., Bauer, R. and Schwitzguébel, J. (2015). Harvesting of *Chlorella sorokiniana* by co-culture with the filamentous fungus *Isaria fumosorosea*: A potential sustainable feedstock for hydrothermal gasification. *Bioresource Technology* **185, 353-361.**

Maza, D., Viñarta, S. C., Su, Y., Guillamón, J. M. and Aybar, M. J. (2020). Growth and lipid production of *Rhodotorula glutinis* R4, in comparison to other oleaginous yeasts. *Journal of Biotechnology* **310, 21-31.**

McGinnis, K. M., Dempster, T. A. and Sommerfeld, M. R. (1997). Characterization of the growth and lipid content of the diatom *Chaetoceros muelleri*. *Journal of Applied Phycology* **9(1), 19-24.**

Michels, M. H. A., van der Goot, A. J., Vermuë, M. H. and Wijffels, R. H. (2016). Cultivation of shear stress sensitive and tolerant microalgal species in a tubular photobioreactor equipped with a centrifugal pump. *Journal of Applied Phycology* **28, 53-62.**

Moshood, T. D., Nawanir, G. and Mahmud, F. (2021). Microalgae biofuels production: A systematic review on socioeconomic prospects of microalgae biofuels and policy implications. *Environmental Challenges* **5, 100207.**

Ogunkunle, O. and Ahmed, N. A. (2019). A review of global current scenario of biodiesel adoption and combustion in vehicular diesel engines. *Energy Reports* **5, 1560-1579.**

Padmaperuma, G., Kapoore, R. V., Gilmour, D. J. and Vaidyanathan, S. (2018). Microbial consortia: A critical look at microalgae co-cultures for enhanced biomanufacturing. *Critical Reviews in Biotechnology* **38(5), 690-703.**

Patel, A., Antonopoulou, I., Enman, J., Rova, U., Christakopoulos, P. and Matsakas, L. (2019). Lipids

detection and quantification in oleaginous microorganisms: An overview of the current state of the art. *BMC Chemical Engineering* **1, 13.**

Peng, L., Fu, D., Chu, H., Wang, Z. and Qi, H. (2020). Biofuel production from microalgae: A review. *Environmental Chemistry Letters* **18, 285-297.**

Qadeer, S., Khalid, A., Mahmood, S., Anjum, M. and Ahmad, Z. (2017). Utilizing oleaginous bacteria and fungi for cleaner energy production. *Journal of Cleaner Production* **168, 917-928.**

Saenge, C., Cheirsilp, B., Suksaroge, T. T. and Bourtoom, T. (2011). Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. *Process Biochemistry* **46(1), 210-218.**

Sambles, C., Middelhaufe, S., Soanes, D., Kolak, D., Lux, T., Moore, K., Matoušková, P., Parker, D., Lee, R., Love, J. and Aves, S. J. (2017). Genome sequence of the oleaginous yeast *Rhodotorula toruloides* strain CGMCC 2.1609. *Genomics Data* **13, 1-2.**

Shuler, M., Kargi, F. and DeLisa, M. (2017). Bioprocess Engineering: Basic Concepts, 3rd Edn. Prentice Hall, Boston.

Singh, D., Sharma, D., Soni, S. L., Sharma, S., Sharma, P. K. and Jhalani, A. (2020). A review on feedstocks, production processes, and yield for different generations of biodiesel. *Fuel* **262, 116553.**

Singh, N. K. and Dhar, D. W. (2011). Microalgae as second generation biofuel. A review. *Agronomy for Sustainable Development* **31(4), 605-629.**

Su, Y., Mennerich, A. and Urban, B. (2012). Coupled nutrient removal and biomass production with mixed algal culture: Impact of biotic and abiotic factors. *Bioresource Technology* **118, 469-476.**

Walne, P. R. (1970). Studies on the food value of nineteen genera of algae to juvenile bivalves of the genera *Ostrea*, *Crassostrea*, *Mercenaria* and *Mytilus*. Fisheries Investigations Series II, 26(5). Her Majesty's Stationery Office, London.

Wang, Y., Zhang, S., Zhu, Z., Shen, H., Lin, X., Jin, X., Jiao, X. and Zhao, Z. K. (2018). Systems analysis of phosphate-limitation-induced lipid accumulation by the oleaginous yeast *Rhodosporidium toruloides*. *Biotechnology for Biofuels* **11(1), 148.**

Wu, J. Y., Lay, C. H., Chen, C. C. and Wu, S. Y. (2017). Lipid accumulating microalgae cultivation in textile wastewater: Environmental parameters optimization. *Journal of the Taiwan Institute of Chemical Engineers* **79, 1-6.**

Xiong, W., Gao, C., Yan, D., Wu, C. and Wu, Q. (2010). Double CO_2 fixation in photosynthesis-fermentation model enhances algal lipid synthesis for biodiesel production. *Bioresource Technology* **101(7), 2287-2293.**

Xue, F., Miao, J., Zhang, X. and Tan, T. (2010). A new strategy for lipid production by mix cultivation of *Spirulina platensis* and *Rhodotorula glutinis*. *Applied Biochemistry and Biotechnology* **160(2), 498-503.**

Yen, H. W., Chen, P. W. and Chen, L. J. (2015). The synergistic effects for the co-cultivation of oleaginous yeast-*Rhodotorula glutinis* and microalgae-*Scenedesmus obliquus* on the biomass and total lipids accumulation. *Bioresource Technology* **184, 148-152.**

Zeng, Y., Xie, T., Li, P., Jian, B., Li, X., Xie, Y. and Zhang, Y. (2018). Enhanced lipid production and nutrient utilization of food waste hydrolysate by mixed culture of oleaginous yeast *Rhodosporidium toruloides* and oleaginous microalgae *Chlorella vulgaris*. *Renewable Energy* **126, 915-923.**

Zhang, Z., Ji, H., Gong, G., Zhang, X. and Tan, T. (2014). Synergistic effects of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* for enhancement of biomass and lipid yields. *Bioresource Technology* **164, 93-99.**

Zhao, G., Yu, J., Jiang, F., Zhang, X. and Tan, T. (2012). The effect of different trophic modes on lipid accumulation of *Scenedesmus quadricauda*. *Bioresource Technology* **114, 466-471.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538