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Enhanced Lipid Production by Co-cultivation and Co-encapsulation of Oleaginous Yeast *Trichosporonoides spathulata* with Microalgae in Alginate Gel Beads

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Abstract This study attempted to enhance biomass and lipid productivity of an oleaginous yeast *Trichosporonoides spathulata* by co-culturing with microalgae *Chlorella* spp., optimizing culture conditions, and encapsulating them in alginate gel beads. The co-culture of the yeast with microalgae *Chlorella vulgaris* var. *vulgaris* TISTR 8261 most enhanced overall biomass and lipid productivity by 1.6-fold of the yeast pure culture at 48 h and by 1.1-fold at 72 h. After optimization and scale-up in a bioreactor, this co-culture produced the highest biomass of 12.2 g/L with a high lipid content of 47 %. The dissolved oxygen monitoring system in the bioreactor showed that the microalgae worked well as an oxygen supplier to the yeast. This study also showed that the co-encapsulated yeast and microalgae could grow and produce lipid as same as their free cells did. Therefore, it is possible to apply this encapsulation technique for lipid production and simplification of downstream harvesting process. This co-culture system also produced the lipid with high content of saturated fatty acids, indicating its potential use as biodiesel feedstock with high oxidative stability.

Keywords Biodiesel feedstock · Co-culture · Encapsulation · Lipid · Oleaginous yeast

Introduction

Research concerning lipid production by microorganisms was stimulated decades ago mainly because of the impending shortage of crude oil. Some microbial lipids are considered as an alternative raw material for biodiesel, a promising alternative fuel in the future [1]. Compared with the traditional production of vegetable or bean oils, the use of microorganisms to produce lipid has many merits such as shorter life cycle, a reduced requirement for labor, less influence

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by territory and climates, and easier scale up [2]. The microorganisms that can accumulate lipid higher than 20 % of their dry weight are defined as oleaginous microorganisms. The lipid content can vary between 20 and 70 %, and in some case, it can reach 90 % of the dry weight under certain conditions [3].

Among the oleaginous microorganisms, yeasts are most advantageous because they have a relatively fast growth rate and high lipid content. Microalgae are also considered as potential lipid producers because they are capable of producing lipid using sunlight and carbon dioxide. In addition to the pure cultures of oleaginous microorganisms, the co-cultures of them have also been attempted to enhance biomass and lipid productivity [4, 5]. In the co-culture of yeast and microalgae, it was postulated that the microalgae would act as an oxygen generator for the yeast while the yeast would provide the carbon dioxide to the microalgae, and together, they would carry out an improved production of lipid. However, these experiments were carried out only in the shake flasks, and the above hypotheses have not been proven.

It has been known that the high energy input required for harvesting biomass also poses the question of the economic feasibility to commercialize microbial lipid. There are several ways to separate biomass from water such as centrifugation and microfiltration. However, these methods consume exceptionally large amounts of energy. The immobilization technology is an interesting technique to simplify biomass separation from water. The immobilized cells can be harvested by a simple sieving method without involving huge amounts of energy input [6]. This technique has also been exploited in order to pre-concentrate microbial cells [7]. Hence, handling of microbial biomass would become easier and feasible to be implemented on a commercial scale. The most common way to immobilize cells is through gel entrapment method, in which natural polysaccharides such as agars, carrageenans, and alginates are preferably used due to their low toxicity and high transparency [6–10].

The immobilization in alginate gel beads is inexpensive and also easy to carry out and provides extremely mild conditions, so there is a high potential for industrial application [11]. The biomass can be easily separated from alginate by dissolving the beads in sodium carbonate prior to centrifugation [6]. However, immobilizing microorganisms in alginate gel beads has also got critical problems in the fields of mechanical strength and then economic feasibility. Therefore, the stabilization of bead strength is required to prolong use of the beads and increase the economic feasibility of this method. Many procedures have been suggested to increase bead strength such as optimizing immobilization process, adding aluminum nitrate (trivalent cations) and cross-linking with polymers such as chitosan and glutaraldehyde [12–14]. The mass production of uniform alginate gel beads is also essential for the employment of immobilized microorganisms in industrial applications. Lee et al. [15] employed a practical mass production of the alginate gel beads by sound waves induced vibration. This method required low apparatus cost and provided easier and better control of bead size and shape.

Recently, an oleaginous yeast *T. spathulata* JU4-57 was isolated and screened for efficient conversion of crude glycerol into lipid [16]. It was expected that the lipid production from crude glycerol could be further enhanced if this oleaginous yeast was co-cultured with the microalgae. The aim of this study was to enhance biomass and lipid production from crude glycerol by: (1) co-culturing this oleaginous yeast *T. spathulata* with several microalgae *Chlorella* spp.; (2) optimizing the culture conditions; and (3) encapsulating them in alginate gel beads. This was the first known research that attempted to co-encapsulate the yeast and microalgae in alginate gel beads for lipid production and simplification of the downstream harvesting process.

Materials and Methods

Microorganisms and Media

The oleaginous yeast *T. spathulata* JU4-57 isolated from the soils at biodiesel plant of the Prince of Songkla University (Songkhla, Thailand) in 2011 was used in this study. *Chlorella vulgaris* var. *vulgaris* TISTR 8261 was obtained from the Thailand Institute of Scientific and Technological Research (Bangkok, Thailand), and a freshwater microalgae *Chlorella* sp. and a marine microalgae *Chlorella* sp. were obtained from the National Institute of Coastal Aquaculture in the southern region of Thailand.

T. spathulata grown in a liquid medium, containing 10 g/L yeast extract, 10 g/L peptone, and 4 g/L dextrose for 24 h at 28°C and 140 rpm, was used as the yeast seed culture. A modified Chu13 medium used for the microalgae culture contained 0.2 g KNO_3, 0.04 g K_2HPO_4, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.054 g $CaCl_2{\cdot}2H_2O$, 0.01 g Fe citrate, 0.1 g citric acid, 0.036 g $NaHCO_3$, and 1 mL of micro-elements per liter, pH 6.7. The micro-element solution consisted of 2.85 g H_3BO_3, 1.8 g $MnCl_2{\cdot}4H_2O$, 0.02 g $ZnSO_4{\cdot}7H_2O$, 0.08 g $CuSO_4{\cdot}5H_2O$, 0.08 g $CoCl_2{\cdot}6H_2O$, and 0.05 g $Na_2MoO_4{\cdot}2H_2O$ [17]. The microalgae grown in the modified Chu13 medium for 5 days at 28°C and 140 rpm under a 2,000 lux light intensity using cool-white fluorescent lamps with a 16:8 h light and dark cycle were used as the microalgae seed culture.

The crude glycerol waste was obtained from the biodiesel plant of the Prince of Songkla University (Songkhla, Thailand). Its average glycerol content was 40 % with other components consisting mainly of potassium and sodium salts (4–5 %), methanol (1–3 %), non-glycerol organic matter (1.6–7.5 %), and water (36–45 %). The crude glycerol-based medium containing 10 % of the crude glycerol and 0.5 % of ammonium sulfate was used as the production medium. The pH was adjusted to 6.0.

Co-culturing of Oleaginous Yeast with Microalgae

Seed cultures of oleaginous yeast and each microalga (yeast and microalgae at a ratio of 10^6:10^6 cells/mL) were transferred into 250-mL Erlenmeyer flasks containing 50 mL of crude glycerol-based medium. In the yeast pure culture, the inoculum size was 10^6 cells/mL. The flasks were incubated at room temperature and 140 rpm under a 2,000 lux light intensity with a 16:8 h light and dark cycle for 5 days. The influence of the microalgae species, microalgae inoculums size, and light intensity on the biomass and lipid production were investigated. The samples were taken every 12 h for determination of cell counts, pH, cell dry weight, lipid production, lipid content, and glycerol concentration. The cultivation was then scaled up to 2 L in a 5-L stirred-tank bioreactor equipped with a monitoring and control system. The culture pH and dissolved oxygen were monitored with a pH meter (Din, Taiwan) and an oxygen probe (Eutech Instrument, China), respectively. To investigate the effect of the pH control, the pH was maintained at 6.0 automatically using 1.0 M sodium hydroxide or 1.0 M ammonia.

Co-encapsulation of the Yeast with Microalgae

The yeast *T. spathulata* and the selected microalgae were co-encapsulated in alginate gel beads according to the modified procedure of Lam and Lee [6]. The culture broth containing yeast and microalgae was mixed with sterile medium and a 4 % sodium alginate solution at a volumetric ratio of 3:1. The mixture solution was extruded drop-wise, through a flat-end needle, using a peristaltic pump into sterile 2 % (*w/v*) calcium chloride solution and stirred with a magnetic stirrer at 200 rpm. The produced beads had an average diameter of 2.5 mm.

The beads containing the microorganisms were stabilized in calcium chloride solution at 4°C for 2 h and then rinsed with distilled water before use. The co-encapsulated microorganisms were 5 % (*v/v*) inoculated into the culture medium and incubated as describe above.

Analytical Methods

Individual cell counts of yeast and microalgae were determined using a hemocytometer. The maximum specific growth rate, denoted by μ, was estimated by the slope of the tangent drawn to the inflexion of the sigmoid curve which was fitted to the data representing the natural logarithm of the cell concentration against time. The biomass concentration was determined gravimetrically. Samples containing 10 mL culture broth were withdrawn from the flasks and centrifuged at 1,585×*g* for 10 min. The cell pellets were collected, washed twice with distilled water, and then dried at 60°C to constant weight. For the immobilized cells, the beads were solubilized with 2 % (*w/v*) anhydrous sodium carbonate before determination of their cell count and biomass [6]. The lipid was extracted from the dry biomass according to the method of Folch et al. [18]. The dry biomass was ground into a fine powder. The powder was blended with 1 mL chloroform/methanol (2:1), and the mixture was sonicated for 30 min. The process was repeated twice more. The combined solvent was removed by evaporation. It should be noted that the extract may contain, beside lipids, other hydrophobic molecules such as lipoproteins and pigments. Lipid content was expressed in the percentage of the extract in relation to the dry biomass (%*w/w*).

The method for fatty acid methyl esters (FAME) production from extracted lipid involved hydrolysis of the lipid followed by esterification. The fatty acid composition in the FAME was analyzed using a HP6850 gas chromatography equipped with a cross-linked capillary FFAP column (length 30 m, 0.32 mm I.D, 0.25 μm film thickness) and flame ionization detector. The operating conditions were as follows: inlet temperature 290°C; oven temperature initial 210 °C hold for 12 min ramp to 250 °C at 20 °C/min; hold for 8 min and the detector temperature was 300°C. Fatty acids were identified by comparing their retention times with the standards. The glycerol concentration was determined spectrophotometrically [19].

All experiments were performed in triplicates. Analysis of variance was performed using the SPSS software to calculate significant differences in treatment means, and the least significant difference ($p \leq 0.05$) was used to separate the means.

Results and Discussion

Enhancing Lipid Production from Crude Glycerol by the Co-cultivation of *T. spathulata* with Microalgae

The oleaginous yeast *T. spathulata* was co-cultured with the three microalgae including *C. vulgaris* var. *vulgaris* TISTR 8261, a freshwater microalgae *Chlorella* sp., and a marine microalgae *Chlorella* sp. in the crude glycerol-based medium (Fig. 1). The initial yeast cell number in all cultures was fixed at 10^6 cells/mL. The effect of microalgae cells at 10^6 cells/mL on the yeast cell growth was evaluated by observing the yeast cell number in Fig. 1a. It should be noted that, although the initial inoculums in the co-culture was twofold of the yeast pure culture (yeast and microalgae at a ratio of 10^6:10^6 cells/mL), this had little effect on the total biomass at 0 h. At 0 h, the biomass of yeast pure culture was 0.35 g/L and that of the co-culture was 0.43 g/L (only 0.08 g/L increased by the biomass of the microalgae). The co-cultivation with the microalgae did promote the growth of the yeast cells (Fig. 1a). The yeast cells in the

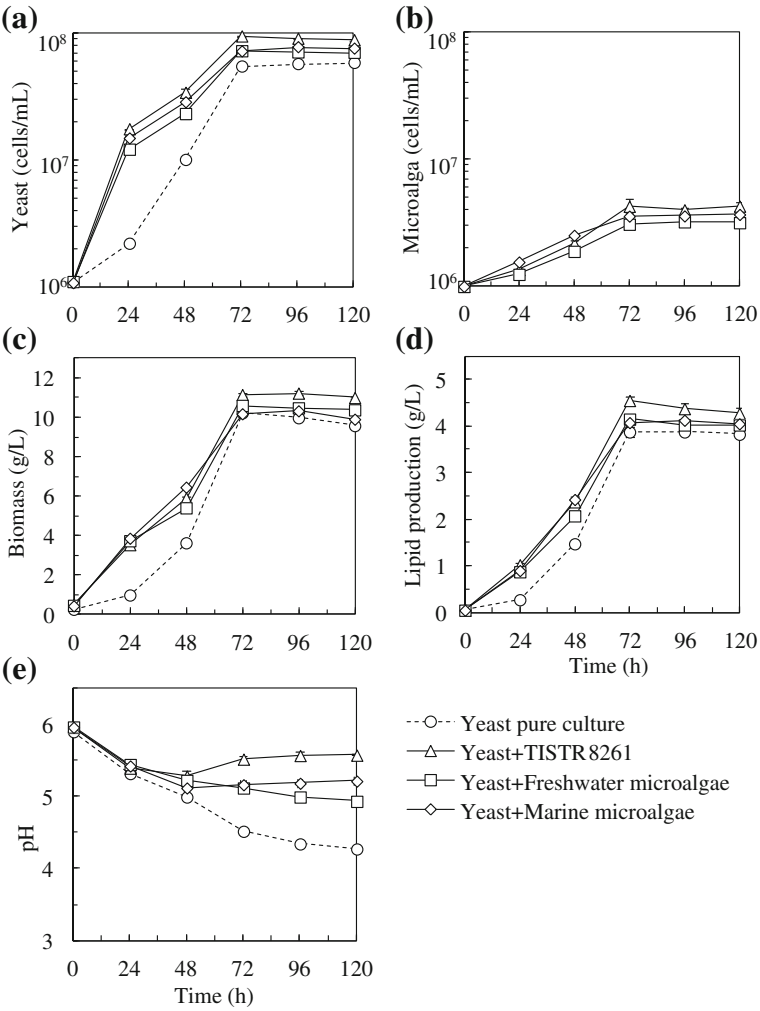

Fig. 1 Comparison of the cell numbers of yeast and microalgae, biomass, lipid, and pH in the pure culture of *T. spathulata* JU4-57 (yeast) and in the co-cultures with *C. vulgaris* var. *vulgaris* TISTR 8261 (yeast+TISTR 8261), a freshwater microalgae *Chlorella* sp. (yeast+freshwater microalgae) and a marine microalgae *Chlorella* sp. (yeast+marine microalgae)

co-culture grew faster than that in the pure culture. The specific growth rates of the yeast in the co-culture (0.067–0.076 h^{-1}) were about 1.45–1.65-fold of that in the pure culture (0.046 h^{-1}). The microalgae grew slower than the yeast with the low specific growth rates of 0.012–0.019 h^{-1} (Fig. 1b). The yeast cells increased from 10^6 cells/mL up to nearly 10^8 cells/mL within 72 h, while the microalgae increased from 10^6 cells/mL only up to 4×10^6 cells/mL. Among the three co-cultures established, the co-culture of the yeast with the microalgae *C. vulgaris* var. *vulgaris* TISTR 8261 produced the highest overall biomass of 11.13 g/L and the highest overall lipid of 4.55 g/L with a corresponding lipid content of 40.81 % (Fig. 1c and d). The overall biomass and lipid productivity of this co-culture at 48 h were 0.125 and 0.052 g/L/h, respectively, which were about 1.6-fold of those of the yeast pure culture (0.079 and 0.031 g/L/h, respectively). However, the biomass and lipid productivity of the co-culture

at 72 h (0.155 and 0.063 g/L/h) were only 1.1-fold of those of the yeast pure culture (0.138 and 0.055 g/L/h). It was possible that, at 72 h, the productivity would be limited by other factors such as depletion of the nutrients.

The results in this study were consistent with the results of Xue et al. [20] who found that a mixed culture of yeast *Rhodotorula glutinis* with the microalgae *Spirulina platensis* gave a higher total lipid production than that obtained either from the pure culture of *R. glutinis* or *S. platensis*. However, the total lipid production in their study was only 0.467 g/L. Cheirsilp et al. [5] studied the mixed culture of the yeast *R. glutinis* with the microalgae *C. vulgaris* in the effluent from a steamed fish process in a seafood processing plant. They also found that the biomass in the mixed culture increased faster than that in the pure culture. However, there was no significant increase in the biomass after 3 days of cultivation. They concluded that this might have resulted from the depletion of the nutrients (nitrogen source) in the culture medium used.

During the yeast pure cultivation, the pH of the culture dropped from 6.0 to 4.3 (Fig. 1e). This could be due to the formation of acids by the yeast metabolism. It was possible that the low pH and these acids might restrain the cell growth of the yeast. In the co-cultures, the pH slightly dropped from 6.0 to 5.2–5.5 and remained steady after 48 h except for the pH in the co-culture with *C. vulgaris* var. *vulgaris* TISTR 8261 that slightly increased after 48 h. Normally, CO_2 dissolves in the water in the form of bicarbonate (HCO^{3-}). When CO_2 is consumed by the microalgae, the OH^- is formed, and the pH becomes more alkaline [21]. Therefore, in the co-culture, the pH might be neutralized by this mechanism. Another possible mechanism would be the acid consumption by the microalgae, since it has been reported that the microalgae could consume some organic acids such as pyruvic and acetic acids [20]. Since it had no big difference in the pH of the pure and co-culture during 48 h, the higher biomass and lipid productivity in the co-culture during this period might not come from the stability of the pH. It was postulated that the microalgae might supply in situ oxygen to the yeast and enhanced the yeast cell growth. However, this phenomenon could not be proven in the shake flask cultivation. In addition, during the co-culture, it was observed that the color of the microalgae were yellow rather than green, which meant that the synthesis of chlorophyll *a* was restrained and a mixotrophic growth process might be conducted by the microalgae using glycerol as an organic carbon source.

Optimizing Lipid Production from Crude Glycerol by the Co-culture

Because the co-culture of the yeast *T. spathulata* with the microalgae *C. vulgaris* var. *vulgaris* TISTR 8261 gave the highest overall biomass and lipid productivity, further optimization of this co-culture was attempted. Since the microalgae grew slower than the yeast (Fig. 1b), it was thought that, if the number of microalgae in the inoculums was increased, the lipid productivity by the yeast might be further enhanced. The initial numbers of microalgae was varied in the range of 10^5–10 cells/mL (Fig. 2). The results were compared with the yeast pure culture. The inoculums size is a fundamental parameter that controls the specific growth rate and the fermentation time. The smaller inocula took longer time to reach the final concentration. On the contrast, the larger inocula led to a rapid increase in the amount of biomass and shortened the fermentation time. The co-culture using the microalgae inoculums size of 10^5 and 10^6 cells/mL increased the specific growth rate of the yeast by 22 % and 73 %, respectively, compared with that of the yeast pure culture (Fig. 2a). However, no further enhancement was observed at larger microalgae inoculums size. The specific growth rate of the microalgae also decreased at a large inoculums size. This could be due to the larger inoculums size would lead to the higher cell density and reduce the light penetration. Although the larger microalgae inocula gave

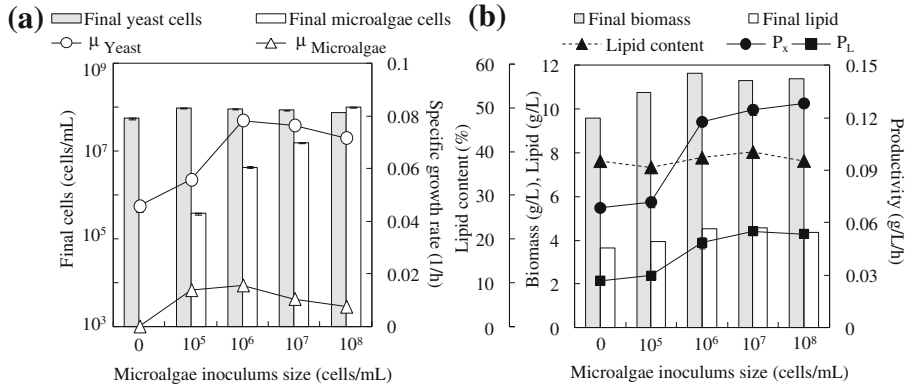

Fig. 2 Effect of microalgae inoculums size on **a** final cell numbers and specific growth rates of yeast (μ_{Yeast}) and microalgae ($\mu_{Microalgae}$) and **b** final concentration and productivity of biomass (P_x) and lipid (P_L)

higher final cells of the microalgae, there was no significant effect on the final cells of the yeast. Figure 2b shows the effect of microalgae inoculums size on the final concentration and productivity of biomass (P_X) and lipid (P_L). Increasing the microalgae inoculums size up to 10^6 cells/mL did increase the productivity of biomass and lipid as well as their final concentrations. It can be concluded that the minimum inoculums size of the microalgae that was effective for stimulating the yeast cell growth and enhancing the biomass and lipid productivity was 10^6 cells/mL. The lipid contents in each co-culture were not much different, and they were in the range of 38–40 %.

Light intensity has an important role on the photosynthetic activity of microalgae. The effect of light intensity on the co-culture was then investigated (Fig. 3). The microalgae grew faster with increasing the light intensity up to 4,000 lux but slightly decreased when the light intensity was further increased to 8,000 lux. This could be due to the photoinhibition effect on the microalgae growth. High specific growth rate of the microalgae also induced high specific growth rate of the yeast (Fig. 3a). Without the light illumination, the microalgae grew very slow and resulted in low final microalgae cells. The biomass and lipid productivity also showed the same trend as found in the specific growth rate (Fig. 3b). The overall biomass and lipid production reached their maximum levels of 11.85 and 4.93 g/L, respectively, at the

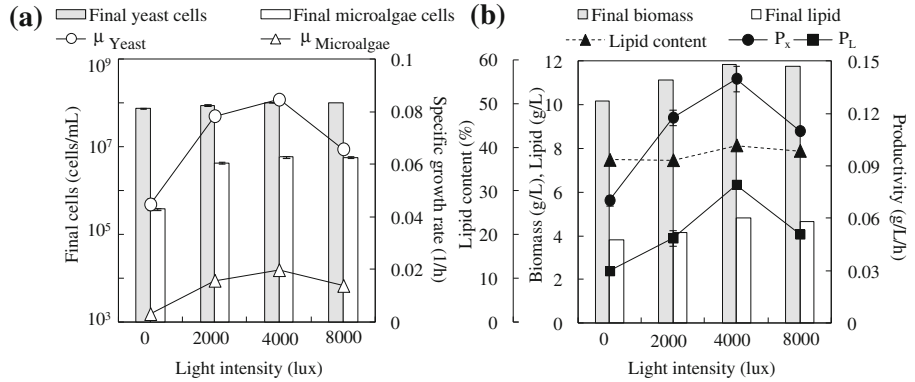

Fig. 3 Effect of light intensity on **a** final cell numbers and specific growth rates of yeast (μ_{Yeast}) and microalgae ($\mu_{Microalgae}$) and **b** final concentration and productivity of biomass (P_x) and lipid (P_L)

light intensity of 4,000 lux. The maximum productivity of biomass and lipid were 0.132 and 0.065 g/L/h, respectively.

The separate pure culture of the yeast *T. spathulata* and the microalgae *C. vulgaris* var. *vulgaris* TISTR 8261 under optimized conditions (microalgae inoculums size at 10^6 cells/mL and light intensity at 4,000 lux) were also tested (data not shown). It was found that the separate pure culture of the yeast and the microalgae produced the biomass of 10.23 and 0.75 g/L, respectively. The sum of these two microorganisms was 10.98 g/L, which was lower than that of the co-culture (11.85 g/L). The sum of lipid produced by the separate pure cultures (4.14 g/L) was also lower than that of the co-culture (4.93 g/L). The results also showed that *C. vulgaris* var. *vulgaris* TISTR 8261 could use glycerol as a carbon source, which suggested that this microalgae could be cultivated either heterotrophically or mixotrophically using organic compounds as energy and carbon sources.

Scale-Up of the Pure Culture and Co-culture in 5-L Stirrer Bioreactor

The pure culture and co-culture of the yeast with the microalgae were scaled-up in a 5-L stirrer bioreactor tank with a working volume of 2 L. Figure 4 shows time courses of cell growth, lipid production, lipid content, and glycerol consumption. The dissolved oxygen (DO) was monitored using a DO probe. The agitation speed was controlled at 100 rpm. The light intensity for the microalgae and the co-culture was 4,000 lux with the light and dark cycle of 16:8 h. The co-culture (Fig. 4c) showed a shorter lag phase and gave a higher overall

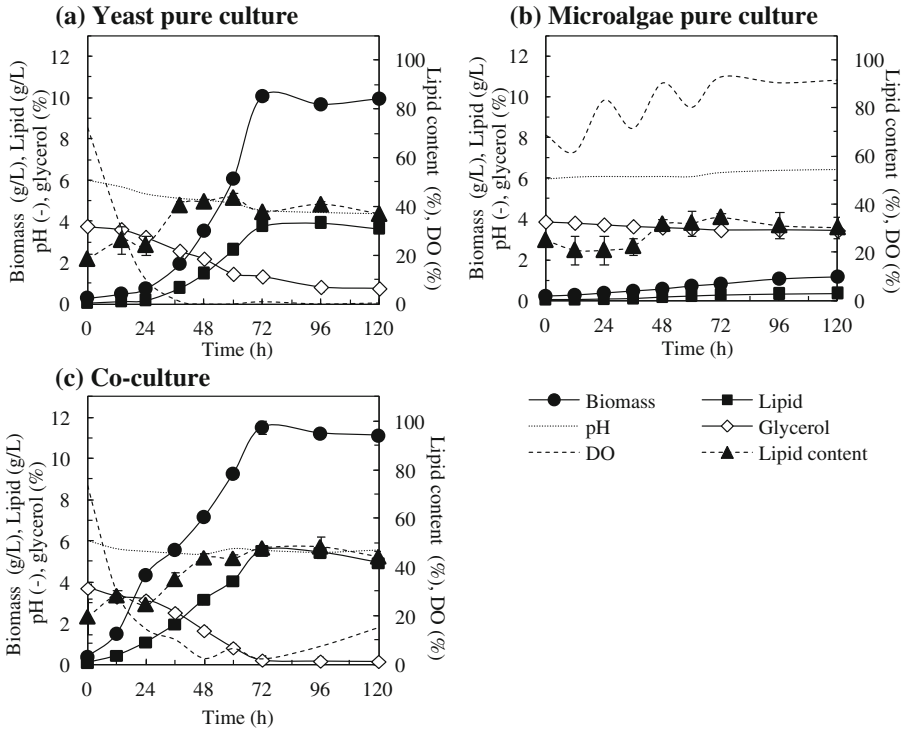

Fig. 4 Performance of the pure culture and co-culture in the bioreactor. **a** The pure culture of *T. spathulata* JU4-57, **b** the pure culture of *C. vulgaris* var. *vulgaris* TISTR 8261, and **c** the co-culture

biomass (11.53 g/L) and lipid (5.8 g/L) compared with the yeast pure culture (Fig. 4a, 10.01 and 3.77 g/L). The biomass and lipid productivity of the co-culture were 0.149 and 0.069 g/L/h, respectively, which were about 2.5-fold of those in the yeast pure culture. It should be also noted that the biomass and lipid productivity did improve when the co-culture was scaled-up in the bioreactor. The glycerol consumption in the co-culture was also faster than that in the yeast pure culture. Although the microalgae could grow on crude glycerol (Fig. 4b), the production of biomass and lipid were much lower than those of the yeast. Interestingly, the lipid content of the co-culture during late log phase (44-49 %) was higher than those of the yeast pure culture (40–43 %) and microalgae pure culture (31–34 %).

In the yeast pure culture, the DO decreased rapidly to almost zero at 48 h due to the continuous consumption of oxygen by the yeast (Fig. 4a) whereas, in the microalgae pure culture, the DO increased and decreased within 24-h cycle (Fig. 4b). This was because the microalgae released oxygen during light illumination period for 16 h and consumed it during dark period for 8 h. It should be noted that the cultivation of microalgae provided the DO as high at 70–90 %. This result has proved that the microalgae could function well as an oxygen producer, and this would be one possible explanation for the promoted yeast growth in the co-culture. The specific growth rate of the microalgae also increased when it was co-cultured with the yeast (data not shown). Therefore, the relationship of the yeast and microalgae would be symbiotic, as the yeast produced CO_2 that could be used by the microalga. In the co-culture, both the two metabolic reactions of CO_2 release and uptake were combined and complementary. In the yeast pure culture, the pH declined from 6.0 to 4.31 (Fig. 4a) possibly due to the production of organic acids by the yeast metabolism. By contrast, the pH in the microalgae pure culture slightly increased from 6.0 to 6.43 (Fig. 4c). In the co-culture, the pH declined during 48 h and slightly increased thereafter. It was possible that the acidic pH resulted from the yeast metabolites might be neutralized by the microalgae metabolism. However, to prevent the inhibition effect by acidic pH during 48 h of the cultivation, the pH control at the constant level was attempted.

The effect of pH control using sodium hydroxide and ammonia was investigated in the bioreactor (Fig. 5). The pH was automatically controlled at 6.0 during 48 h of the cultivation. With the pH control using sodium hydroxide (Fig. 5a), the cell numbers of the yeast increased from 10^6 cells/mL up to 8.3×10^7 cells/mL, and the cell numbers of the microalgae slightly increased from 10^6 cells/mL up to 4.94×10^6 cells/mL whereas, with the pH control using ammonia (Fig. 5b), the cell numbers of the yeast and microalgae increased up to 1.18×10^8 cells/mL and 5.89×10^6 cells/mL, respectively, which were slightly higher than those using sodium hydroxide. It was likely that both the yeast and microalgae might use ammonia as an extra nitrogen source for their cell proliferation. The overall biomass and lipid in the co-culture with pH control using ammonia were also slightly higher (12.2 and 5.74 g/L, respectively) than those using sodium hydroxide (11.3 and 5.21 g/L, respectively). The biomass and lipid productivity of the co-culture with pH control using ammonia were 0.159 and 0.079 g/L/h, respectively, and those using sodium hydroxide were 0.146 and 0.074 g/L/h, respectively. Although the overall biomass in the co-culture with pH control was slightly improved, the positive effect on lipid production was less than expected.

Immobilization Technique for Enhancing Lipid Production and Simplifying the Downstream Process

The immobilization technique can simplify the separation of the microorganism biomass separation from liquid medium. After the immobilized microorganism beads have grown to stationary phase, the beads can be easily harvested through a simple sieving method without involving huge amounts of energy input. It was also expected that the co-encapsulation of

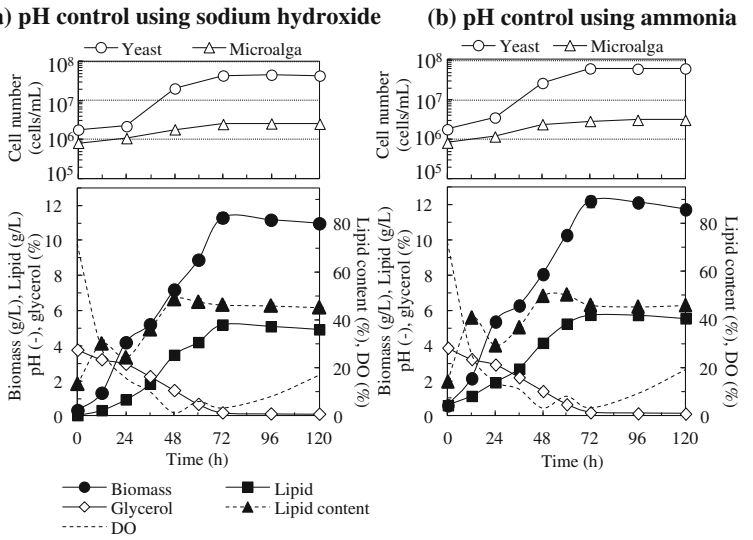

Fig. 5 Effect of the pH control using sodium hydroxide (**a**) and ammonia (**b**) on the co-culture of *T. spathulata* JU4-57 and *C. vulgaris* var. *vulgaris* TISTR 8261

these two strains in the cavity of the gel beads would more enhance the interaction between the two strains or at least alleviate the problem of the gas transfer limitation. This is because the microalgae could act as an oxygen generator for the yeast while the yeast could provide CO_2 to the microalgae, and together, they would be able to carry out biomass and lipid production inside the gel beads.

In this study, the mixed microorganisms were immobilized in alginate gel beads and 5 % (*v*/*v*) inoculated in a 250-mL flask containing 50 mL crude glycerol-based medium. The performance of the immobilized beads is shown in Fig. 6a. The yeast and microalgae grew well in the alginate gel beads as same as their free cells did. The maximum cell mass reached in the beads was 0.78 mg/bead. The pH in the medium declined slightly from 6.02 to 5.53. The maximum cumulated biomass and lipid per liter of the culture medium were 9.48 and 3.84 g/L, respectively, with a corresponding lipid content of 40.4 %. After 3 days of cultivation, uncontrollable free cells were released from the beads into the medium. Lam and Lee [6] also reported that uncontrollable free cells were detected in their cultures due to leakage from the beads. It was possible that the alginate gel beads might become over-saturated with the cells, and part of the cells escaped into the medium, leading to the uncontrollable growth of a free cell culture. To avoid this consequence, the beads should be harvested immediately after entering early stationary phase.

The co-culture using immobilized cells was also scaled-up in a 5-L bioreactor (Fig. 6b). The DO was monitored using the DO probe. The DO decreased rapidly from 70 % to nearly 0 % at 48 h, which was similar trend in the co-culture of the free cells. The cumulated biomass and lipid per liter of the culture medium were 9.13 and 3.75 g/L, respectively. The biomass and lipid productivity using encapsulated co-culture were 0.134 and 0.050 g/L/h in the shake flasks and 0.132 and 0.049 g/L/h in the bioreactor. Although the performances of the encapsulated cells were slightly lower than those of the free cells, the encapsulated cells were easily harvested using sieving method. This procedure could be a promising way to reduce the downstream harvesting costs and contribute greatly to the industrialization of microbial lipid production.

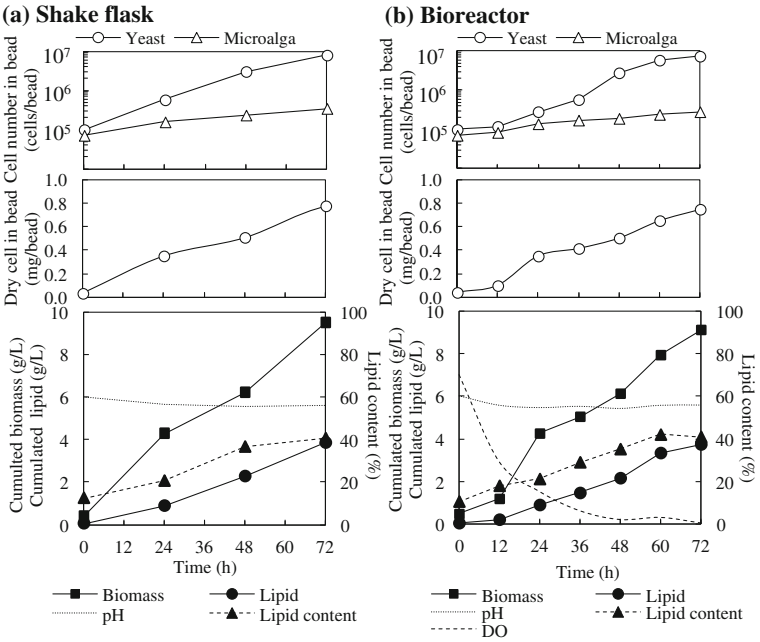

Fig. 6 Performance of the immobilized co-culture of *T. spathulata* JU4-57 with *C. vulgaris* var. *vulgaris* TISTR 8261 in shake flasks (**a**) and the bioreactor (**b**)

Lipid Composition

Table 1 shows the fatty acid composition of the lipid extracted from yeast *T. spathulata*, microalgae *C. vulgaris* var. *vulgaris* TISTR 8261, and their co-culture growing on crude glycerol. The lipids obtained in this study were mainly composed of long-chain fatty acid with 16 and 18 carbon atoms. The lipid from the yeast was mainly composed of oleic acid (C18:1, 48.91 %) followed by linoleic acid (C18:2, 19.16 %) and palmitic acid (C16:0, 17.96 %). The lipid from the microalgae was also composed of oleic acid (37.49 %) as the predominant fatty acid but followed by palmitic acid (32.32 %) and linoleic acid (15.69 %). The fatty acid composition of the yeast lipid in this study was consistent with other results in the literature such as *R. glutinis* [22], *Rhodosporidium toruloides* Y4 [23], and *Rhodotorula mucilaginosa* TJY15a [24] that contained mainly oleic acid as the predominant fatty acid. Bellou et al. [25] reported that several strains of zygomycetes cultivated on glycerol accumulated lipid especially polyunsaturated fatty acids along with mycelia growth. The fatty acid composition of the microalgal lipid in this study

Table 1 Fatty acid composition of yeast and microalgal lipid produced from crude glycerol

Lipid source	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C22:1	SFA	USFA	Others
Yeast	17.96	0.87	3.93	48.91	19.16	4.63	0.38	21.89	73.95	4.54
Microalga	32.32	2.31	8.27	37.49	15.69	2.94	0.00	40.47	58.43	1.10
Mixed culture	40.52	1.15	17.15	21.3	1.41	0.00	8.89	57.67	32.75	9.58

SFA saturated fatty acid, *USFA* unsaturated fatty acid

was also similar to those in the literature which contained oleic acid and palmitic acid as the predominant fatty acids [26–28].

In contrast to the lipid from the two pure cultures, the lipid from the co-culture composed of palmitic acid (40.52 %) as the predominant fatty acid followed by oleic acid (21.30 %) and stearic acid (17.15 %). Therefore, the lipid from the co-culture contained higher saturated fatty acids (palmitic acid and stearic acid) than those of the two pure cultures. It should be noted that the contributions to lipid production in the co-culture were different between the two microorganisms. Because the yeast dominated the co-culture in terms of cell number and cell dry weight, therefore, it is reasonable to postulate that the lipid would mainly come from the yeast rather than the microalga. Thus, the change in the fatty acid composition might result from the composition change in the yeast lipid under the co-culture condition. It was also possible that the in situ oxygen supply by microalgae might stimulate the synthesis of saturated fatty acids (palmitic acid and stearic acid). Cai et al. [29] also reported that the mixed culture of the microalgae *Isochrysis galbana* 8701 and the yeast *Ambrosiozyma cicatricose* was richer in saturated fatty acids than the two pure cultures. The fatty acid compositional profile in this study was similar to that of plant oil which contains mainly palmitic and oleic acids. This indicates that the microbial lipid from this co-culture have the potential for use as a biodiesel feedstock. Although the high content of saturated fatty acid (palmitic acid) would provide for lower fuel properties at low temperatures, it would provide better oxidative stability.

Conclusions

The co-cultivation of oleaginous yeast *T. spathulata* with microalgae *C. vulgaris* var. *vulgaris* TISTR 8261 increased the biomass and lipid productivity from crude glycerol. Thus, using this co-culture system to reproduce the biodiesel from crude glycerol would provide an added bonus to offset the production costs. This study also showed the feasibility of using encapsulation technique to simplify the biomass separation by sieving method and hence reduce the harvesting costs. This yeast and microalgal lipid has a composition similar to that of plant oil, indicating its possible use as biodiesel feedstock.

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References

1. Xue, F., Zhang, X., Luo, H., & Tan, T. (2006). *Process Biochemistry, 41*, 1699–1702.
2. Li, Q., & Wang, M. Y. (1997). *Science and Technology of Food Industry, 6*, 65–69.
3. Spolaore, P., Joannis-Cassan, C., Duran, E., & Isambert, A. (2006). *Journal of Bioscience and Bioengineering, 101*, 87–96.
4. Cheirsilp, B., Kitcha, S., & Torpee, S. (2012). *Annals of Microbiology, 62*, 987–993.
5. Cheirsilp, B., Suwannarat, W., & Niyomdecha, R. (2011). *New Biotechnology, 341*, 1–7.
6. Lam, K. M., & Lee, K. T. (2012). *Biochemical Engineering Journal, 191*, 263–268.
7. Carrilho, E. N. V. M., No'brega, J. A., & Gilbert, T. R. (2003). *Talanta, 60*, 1131–1140.
8. Cao, Y., Liu, Z., Cheng, G., Jing, X., & Xu, H. (2010). *Chemical Engineering Journal, 164*, 183–195.
9. Moreira, S. M., Moreira-Santos, M., Guilhermino, L., & Ribeiro, R. (2006). *Enzyme and Microbial Technology, 38*, 135–141.

10. Garrido, I. M. (2008). *Bioresource Technology, 99*, 3949–3964.
11. Nussinovitch, A. (2010). *Polymer macro-and micro-gel beads: Fundamentals 27 and applications* (pp. 27–52). New York: Springer Science.
12. Roca, E., Meinander, N., Núñez, M.J., Hahn-Hägerdal, B. and Lema, J.M. (1996). In: Progress in biotechnology—Immobilized cells—Basics and applications (pp. 173–180). The Netherlands: Elsevier B.V.
13. Winkelhausen, E., Velickova, E., Amartey, S. A., & Kuzmanova, S. (2010). *Applied Biochemistry and Biotechnology, 3*, 2214–2220.
14. Zhou, Z., Li, G., & Li, Y. (2010). *International Journal of Biological Macromolecules, 3*, 21–26.
15. Lee, H. H., Park, O. J., Park, J. M., & Yang, J. W. (1996). *Journal of Chemical Technology and Biotechnology, 67*, 255–259.
16. Kitcha, S., & Cheirsilp, B. (2013). *Bioenergy Research, 6*(1), 300–310.
17. Tansakul, P., Savaddiraksa, Y., Prasertsan, P., & Tongurai, C. (2005). *Thai Journal of Agricultural Science, 38*, 71–76.
18. Folch, J., Lees, M., & Stanley, G. H. S. (1957). *Journal of Biological Chemistry, 226*, 497–509.
19. Kosugi, Y., Takahashi, K., & Lopez, C. (1995). *Journal of American Oil Chemistry Society, 72*, 1281–1285.
20. Xue, F., Miao, J., Zhang, X., & Tan, T. (2010). *Applied Biochemistry and Biotechnology, 160*, 498–503.
21. Richmond, A. (1986). *Handbook of microalgal mass culture*. Boca Raton Florida: CRC Press, Inc.
22. Saenge, C., Cheirsilp, B., Suksaroge, T. T., & Bourtoom, T. (2011). *Process Biochemistry, 46*(1), 210–218.
23. Li, X., Xu, H., & Wu, Q. (2007). *Biotechnology and Bioengineering, 98*, 764–771.
24. Li, M., Liu, G. L., Chi, Z., & Chi, Z. M. (2010). *Biomass and Bioenergy, 34*, 101–107.
25. Bellou, S., Moustogianni, A., Makri, A., & Aggelis, G. (2012). *Applied Biochemistry and Biotechnology, 166*, 146–158.
26. Gao, C., Zhai, Y., Ding, Y., & Wu, Q. (2010). *Applied Energy, 87*, 756–761.
27. Converti, A., Casazza, A. A., Ortiz, E. Y., Perego, P., & Borghi, M. D. (2009). *Chemical Engineering and Processing, 48*, 1146–1151.
28. Petkov, G., & Garcia, G. (2007). *Biochemical Systematics and Ecology, 35*, 281–285.
29. Cai, S. Q., Hu, C. Q., & Du, S. B. (2007). *Journal of Bioscience and Bioengineering, 104*, 391–397.