Agricultural biowaste, rice bran, as carbon source to enhance biomass and lipid production: analysis with various growth rate models

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ABSTRACT

As a byproduct of agriculture, rice bran can be a good alternative carbon source to mass-produce microalgae and increase lipid content. The purpose of this study was to investigate the effects of rice bran extract (RBE) on the mass culture and oil content of microalgae. Various parameters were applied to the growth rate model to explain the dynamics of substrate inhibition and growth of microalgae. The rice bran contains 46.1% of carbohydrates, in which is 38.3% glucose, and is very suitable as a carbon source for microalgae growth. The culture with RBE had a four times higher biomass production than microalgae cultured on Jaworski's medium (JM) with a small amount of 1 g/L. In addition, for RBE, the lipid content was three times higher and saturated fatty acid was 3% lower than were those of JM. According to the above results, when *Chlorella vulgaris* is cultured using RBE, a high amount of biomass and high lipid content can be obtained with a small amount of RBE. RBE is a discarded waste and has a high content of glucose, so it can be replaced by an organic carbon source to increase microbial biomass growth and lipid content at low cost.

Key words | biomass, carbon sources, kinetic model, lipid content, microalgae, rice bran

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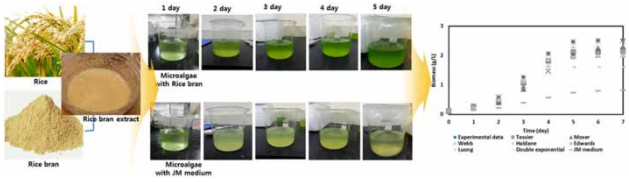
HIGHLIGHTS

- Rice bran extract (RBE) was used to enhance biomass and lipid production of microalgae.
- 1 g/L was the optimum amount of RBE for increasing biomass and lipid contents.
- Lipid content increased three times more by RBE than by Jaworski's medium.
- Biomass production by RBE was four times higher than Jaworski's medium.
- RBE is a discarded waste and has a high glucose content.

GRAPHICAL ABSTRACT



INTRODUCTION

Due to limited oil reserves, unstable oil prices, increased global warming, and concerns about nuclear energy, the search for and development of new alternative energy sources has become a globally important issue. Of the various alternatives, microalgae-derived biomass is emerging as a new, sustainable, and clean energy resource for the

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production of third generation biofuels (Wang *et al.* 2017). Almost all of the microalgae resemble mono-cellular photosynthetic organisms that live in salt or fresh water and are often called 'phytoplankton' (Choi 2014). Because microalgae are very common organisms that are widely distributed throughout the planet, they form the lowest layer of the food chain of the marine ecosystem (Choi & Lee 2014). The greatest advantage of using microalgae as a new alternative energy source is that cell division is very easy and populations can increase numerous times in a short period of time (Choi & Lee 2015a). The production of corn and sugarcane, the main raw materials for biofuels, cannot be doubled in a short period of time. However, microalgae can rapidly multiply if the environmental conditions such as water temperature, light, and nutrients are optimal (Gupta *et al.* 2015). In addition, unlike ordinary plants, microalgae do not require stem or root tissue, and all cells can thus participate in photosynthesis. Thus, the amount of biofuel obtained from microalgae when cultivated in the same area is much higher than that obtained from plants such as corn and soybeans (Gupta *et al.* 2016c). Finally, microalgae do not generate increasing costs because they do not need to be cultivated in a cropland. The use of microalgae as a biofuel does not pertain to the current controversy about using precious food sources for fuel, such as with biofuels sourced from crops. Since microalgae grow in water environments, the land does not need to be used for cultivating, leaving the land available for growing food crops (Choi & Lee 2015a; Choi & Yu 2015). Despite these many advantages, the principal obstacle to the commercialization of microalgae-derived biodiesel is the over-production cost compared to fossil fuels. In order to lower the cost of micro-algae-derived biodiesel, various factors need to be considered such as the selection of microalgae strains with high lipid content, cultivation method, and harvesting method (Gupta *et al.* 2016a). Among these, the problem of mass cultivation of microalgae using an inexpensive carbon source is the first problem that needs to be addressed.

Microalgae are photosynthetic organisms with relatively simple growth requirements other than water, carbon dioxide, light, and nutrients. They also have explosive growth potential when growth conditions are optimized (Gupta *et al.* 2016b). Previous studies have shown that microalgae can cultivate under autotrophic, mixotrophic, and heterotrophic conditions (Gupta *et al.* 2016a). When microalgae are grown under heterotrophic or mixotrophic conditions, the biomass can be harvested 4–5 times and the lipid content in the microalgae cells can be increased more than 40% compared to the autotrophic condition (Li *et al.* 2014; Wang *et al.* 2017). The commonly used carbon sources for mass culture of microalgae include xylose, saccharide, acetate, glucose, and methanol (Li *et al.* 2014). Among these, glucose is the most commonly used. However, any external carbon source requires additional costs, and the cost of such an external carbon source mitigates the price reduction of microalgae-derived biodiesel. In this study, in order to overcome this problem, rice bran extract (RBE) was used, which is a discarded agricultural waste containing a large number of minerals, sugars, and nutrients, as an external carbon source for the mass culture of microalgae and to increase the lipid content.

Rice is one of the world's three major crops, along with corn and wheat, and is an important food source in Asian countries (Signes-Pastor *et al.* 2017). Rice bran forms from peeling rice husk, which is a crumbly mixture of peel, seed coat, hornblende, and embryo. This rice bran contains 95% of the nutrients contained in rice (Zhao *et al.* 2018). The annual production of rice bran in Korea is about 400,000 tons, of which 3% is used for livestock feed, 5% is used for rice bran oil, and 92% is discarded (Hong & Wang 2017). Rice bran contains 38.3% carbohydrates as well as inorganic nutrients and minerals (Hong & Wang 2017; Signes-Pastor *et al.* 2017; Zhao *et al.* 2018). Therefore, it could be considered as an important external carbon source for microalgae mass cultivation. The purpose of this study is to investigate the effects of rice bran extract (RBE) on *Chlorella vulgaris (C. vulgaris)* growth and lipid content in the mixotrophic condition. The effects of the pH, temperature, and concentration of rice bran concentrate on microalgae were analyzed using the Haldane, Double exponential, Edwards, Luong, Webb, Moser and Teissier models. In addition, the lipid content of the microalgae grown in the RBE was analyzed and compared with microalgae cultured in Jaworski's medium (JM).

MATERIALS AND METHODS

Cultivation of *Chlorella vulgaris*

In this study, *Chlorella vulgaris* (*C. vulgaris:* KMMCC 145) was selected, which is high in lipid content and resistant to environmental change compared to other microalgae (Choi 2014). *C. vulgaris* has a high protein content, a balanced amino acid composition, and the potential to produce high value-added products useful in various fields such as pharmaceuticals, health foods, feeds, and nutrients (Choi & Lee 2014). The microalgae used in the experiment were

purchased from the Korea Ocean Research & Development Institute (KAERI) at a quantity of 15 mL and were grown in a thermostat at 25 ± 1 °C for 5 days using JM. The components of the detailed JM are described in Choi & Yu (2015). The light source of the incubator was a light emitting diode (LED) and the model FP-60-12 power supply (AD & Lighting, Suwon, Kyonggi-Do, Korea) was used for the microalgae cultivation. The LEDs were white in color, with a light intensity of 120 μmol/m^2s; the light period was 16 L: 8D at pH 7. The initial concentration of *C. vulgaris* in the control without RBE was 0.1124 ± 0.02 g/L.

Rice bran extract

Rice bran was collected at a farm in Gangneung, Korea. The collected rice bran was filtered using an 80 mesh sieve to remove the rice husk and contaminants. Rice bran under an 80 mesh sieve was dried in an oven at 80 °C for 24 hours to sterilize the microorganisms. 50 g of dried rice bran was added to 1 L of distilled water and stirred at 60 °C in a shake incubator (MR-R505, Mrga Science, Korea) to extract the main nutrients contained in the rice bran. The RBE was filtered with a membrane filter of 0.45 μm (Whatman) to separate the residue. The RBE was refrigerated at less than 4 °C, diluted to a predetermined concentration, and used for microalgae growth experiments.

Experimental design

The experiment was carried out in the form of a batch-test, and the temperature, photo period, and light intensity were set to the same conditions as the culture conditions. Microalgae and various amounts (0–20 g/L) of RBE were mixed in a 10 L reactor according to the experimental design. The mixed solution was reacted at a stirring speed of 150 rpm for 7 days. After the reaction was completed, 10 mL of the solution was sampled at a predetermined time, and the biomass increase was measured. The pH was adjusted to 3–12 with 0.5 mol NaOH and HCl and the temperature was adjusted to 15–40 °C. All experiments were carried out with all other parameters fixed during the measurement of each parameter.

Analytical methods

Qualitative and quantitative analyses of inorganic elements and metals contained in RBE were performed using an inductively coupled plasma-optical emission spectrometer (700 ICP-OES, Aglient Technologies, USA). The RBE component analysis was performed using liquid chromatography (Agilent 1290; Agilent Technologies, USA). The specific growth rate (μ) was calculated using Equation (1)

$$\mu = \mathrm{In}\left(\frac{X_1 - X_0}{t_1 - t_0}\right) \quad (1)$$

where μ is the specific growth rate (1/day), x_0 and x_1 are the maximum concentration of microalgae (cells/mL) after the initial and constant incubation time, respectively, and t is the incubation time (day).

The dry mass of microalgae in biomass was measured by filtering 50 mL of the sample with a GF/C grade microfiber/glass filter (Whatman, UK) and drying at 105 °C for 24 hours.

$$B = \frac{C_b - C_{b_0}}{t - t_0} \quad (2)$$

where B is the amount of biomass, and C_b and C_{b0} are the amounts of biomass at t and t_0, respectively. The growth and inhibitory effects of microalgae on various conditions were analyzed using the Haldane, Double exponential, Edwards, Luong, Webb, Moser and Teissier models. pH was measured using a pH meter (ISTEK, pH-20N).

A wet oil extraction method was used, and the solvent was a nucleic acid (Junsei, Japan). The microalgae mixed with the nucleic acid were stirred for 3 hours, and the solvent-containing solvent layer was recovered and evaporated under reduced pressure (Genevac, EZ2 PLUS). The solvent was volatilized and the remaining oil was recovered. The above extraction procedure was repeated three times for complete recovery of the oil. The amount of microalgae oil extracted was calculated as follows:

$$\text{Lipid content} = \frac{\textit{Amount of recovered oil (g)}}{\textit{Amount of dried microalgae (g)}} \times 100 \quad (3)$$

Analysis of fatty acids in microalgae was carried out according to EN ISO 5508 (EN ISO 5508) and EN ISO 550 after analysis of lipid content (mg/g oil) of extracted oils.

Statistical analysis

The data presented in the tables and figures are the mean values ±3.σ of five replications. Where error bars are not visible, the errors were smaller than or equal to the symbols. The differences between the mean values were calculated using Tukey's test at the 0.05 level with Origin software (v.7.5, OriginLab, Northampton, MA, USA).

RESULTS AND DISCUSSION

Characteristic of the rice bran extract

The RBE contains about 13.5% moisture, 13.2% protein, 18.3% lipid, 8.9% ash, and 46.1% carbohydrate. It also contains a large number of minerals including Ca, P, Fe, Zn, and Mg, which are essential for the growth of microalgae. In particular, P and K contain 15 and 18 mg/g, respectively. The carbohydrates contained in RBE are 38.3% of glucose and 7.8% of cellulose (Table 1). According to previous studies, mainly glucose is used as an external carbon source for mass proliferation of microalgae (Hong & Wang 2017). Glucose improves microbial photosynthesis and the absorption of organic matter (Zhao *et al.* 2018). RBE contains large amounts of glucose and essential minerals, which is believed to have a significant influence on microalgae growth.

Impact of various parameters on the growth of microalgae

pH

pH determines the solubility of CO_2 and minerals in microalgae media and directly or indirectly affects the metabolism of microalgae (Chiu *et al.* 2015). To investigate the effect of pH on the microbial mass culture using RBE, the temperature was controlled at 25 °C, the concentration of RBE extract was 2 g/L, and the pH was adjusted to 3–10. Experiments were carried out for 5 days using *C. vulgaris* at various pH and the growth rate was calculated using average data.

The calculated *C. vulgaris* growth data were applied to various growth models and the results are shown in Figure 1. Experimental results showed that the growth rate of *C. vulgaris* was 0.515–0.601 (day^{-1}) at pH 8, which was the best growth rate. Subsequently, the growth rate decreased with increasing pH. Especially, pH 10 showed a low growth rate of 0.135–0.172 (day^{-1}) and a lower growth rate of 0.01–0.012 (day^{-1}) at pH 12. Thus, at a higher pH of 10, the proliferation of *C. vulgaris* significantly reduced, regardless of whether or not RBE was added. The growth rate of microalgae using JM was similar to that of RBE, and the growth rates were 0.08 and 0.11 (day^{-1}) at pH 7 and 8, respectively. The solubility of inorganic carbon is directly related to the growth of microalgae, and the solubility of inorganic carbon is influenced by pH. Most of the inorganic carbon is present as CO_2 when the pH is less than 5, and CO_2 and HCO_3 are present in the same condition at pH = 6.6 and almost HCO_3 when the pH is 8.3 (Farooq *et al.* 2016; Figueroa-Torres *et al.* 2017). In general, microalgae convert inorganic carbon in the form of CO_2 and HCO_3 into carbon dioxide via carbonic anhydrase and use the carbon for photosynthesis. The microalgae then grow through photosynthesis using these inorganic carbon sources (Cheah *et al.* 2016). Therefore, pH 8 is optimal for growing *C. vulgaris*, and pH 7–9 is the

Table 1 | Nutrient composition of rice bran extracts

Composition		Rice bran extract
Calorie		286 (Kcal)
Moisture		13.5%
Protein		13.2%
Fat		18.3%
Ash		8.9%
Carbohydrate	Glucose	38.3%
	Cellulose	7.8%
Vitamins	Retinol	0 μg/g
	ß-carotene	6.0 mg/g
	Thiamin	2.50 mg/g
	Riboflavin	0.50 mg/g
	Niacin	25.0 mg/g
	Ascorbic acid	0 mg/g
Minerals	Ca	0.8 mg/g
	P	15 mg/g
	Fe	0.6 mg/g
	Na	0.5 mg/g
	K	18 mg/g
	Zn	0.4 mg/g
	Mg	4.0 mg/g
	Cu	0.6 mg/g

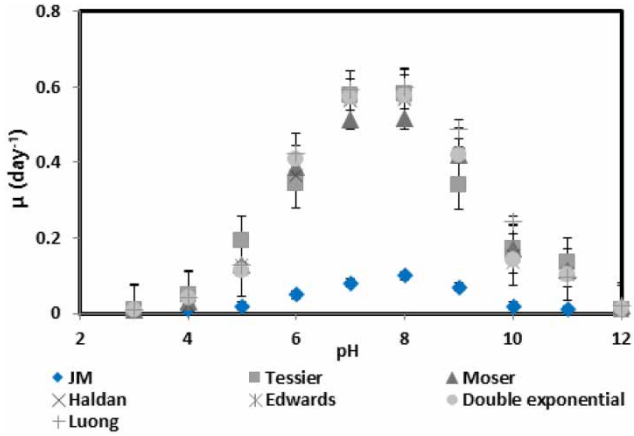

Figure 1 | Maximum specific growth rate of *Chlorella vulgaris* by various pH values with different inhibition models (cultivation: 5 days, amount of rice bran extract: 1 g/L, and temperature: 25 °C, $n = 5$).

optimum range for culturing microalgae. In particular, inorganic carbon is present in the form of CO_3 at up to pH 9, at which it is not absorbed by *C. vulgaris*, and the growth of microalgae is thus inhibited (Heimann 2016). Therefore, in order to increase the absorption and utilization of CO_2 by microalgae, the pH should be controlled during cultivation (Lee *et al.* 2015). According to previous studies, the maximum growth rate (μ_{max}) was 0.382 (day^{-1}) at pH 8 when *C. vulgaris* was cultivated using corncob extract, a biological material (Choi & Lee 2019). The RBE used in this study showed a growth rate ranging from 1.35 to 1.52 times higher than that of corncob extract. This suggests that the carbon sources contained in RBE acted as external nutrients to *C. vulgaris* growth. pH affects the physiological parameters of microalgae because it can directly affect the cell permeability and the hydronium form of the inorganic salt. It can also indirectly affect the absorption of inorganic salts. Therefore, pH plays an important role in the microbial metabolism and control of hydrogen production.

Effect of RBE concentration

While the growth of microalgae requires various nutrients and minerals, P, N, and K are essential nutrients for microalgae growth (Lee *et al.* 2015). However, in general, the amounts of P, N, and K in water are relatively small and are distributed (Markou *et al.* 2014). These conditions often serve as limiting factors for plant growth, including microalgae, and can determine microalgae productivity under conditions where light is sufficient and temperature is optimal (Choi & Lee 2015a; Gupta *et al.* 2016c). The main nutrients of carbon and hydrogen can be obtained from water and air, but nitrogen, phosphorus, potassium, and trace elements must be absorbed from external nutrients (Farooq *et al.* 2016). Therefore, optimizing the concentration of external carbon sources for the mass cultivation of microalgae is an important parameter along with pH and temperature. To investigate the effect of RBE concentration on *C. vulgaris* proliferation, RBE was varied to 0–25 g/L and fixed at pH 7 and 25 °C according to the results of previous experiments. The growth rate of *C. vulgaris* increased rapidly with increasing RBE concentration of up to 1 g/L RBE in all growth models (Figure 2). The growth rate of *C. vulgaris* was the highest at 0.61 (day^{-1}) in the Luong model, followed by the Webb 0.58 (day^{-1}), and Tessier and Edwards 0.58 and 0.57 (day^{-1}) models. In the results of the analysis of experimental data by applying various control models, the *C. vulgaris* growth experiment showed maximum growth rate of 0.60 (day^{-1}) using RBE, and the maximum growth rate using the Luong model was closest to that of the experimental data.

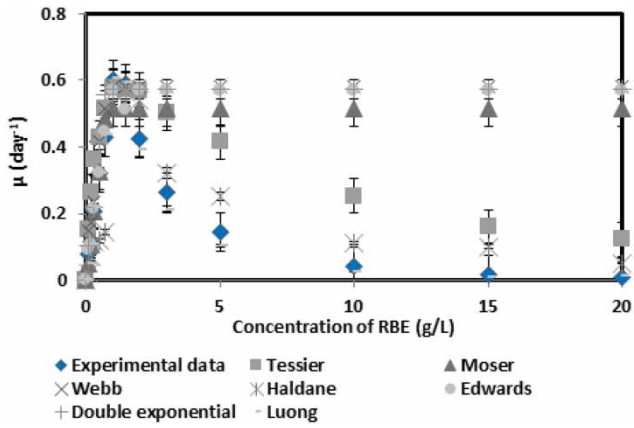

Figure 2 | Maximum specific growth rate by different doses of rice bran extract (pH: 7, temperature: 25 °C, and cultivation: 5 days, $n = 5$).

The various growth control models are directly related to the growth rate of the microalgae and the initial RBE concentration, and can also be used to deduce the substrate saturation coefficient and maximum substrate concentration at which the growth is discontinued (Figueroa-Torres *et al.* 2017). As the concentration of RBE increased above 3 g/L, the growth rate decreased rapidly. Finally, at an RBE concentration of 20 g/L, the growth of microalgae significantly slowed at 0.12–0.58 (day^{-1}). During the experimental period, the color of *C. vulgaris* gradually changed to brown after 3 days with the RBE concentration of 3 g/L, and the *C. vulgaris* grew abnormally at the beginning of the experiment until the RBE concentration of 5 g/L was reached. This suggests that, as the concentration of RBE increased, the content of nutrients also increased, and excessive nutrients adversely affected the growth of *C. vulgaris*. Similar research results was found in previous studies. While microalgae growth was increased when glucose was below 10 g/L, it was inhibited above 10 g/L. In addition, excessive quantities of nutrients caused the active growth of fungi in the medium (Lee *et al.* 2015; Geada *et al.* 2017). Choi & Lee (2019) reported that *C. vulgaris* growth was stopped when the corncob extract reached 15 g/L, and white fungi appeared on the medium after 2 days. From the results of this experiment, white fungus appeared at an RBE concentration of 5 g/L after 3 days. From day 4, the proliferation of the fungus increased sharply, and the proliferation of *C. vulgaris* was markedly decreased when viewed with the naked eye. These results indicate that as the concentration of RBE increases, the fungi appear earlier and proliferate more actively. The organic ingestion of

microalgae occurs through sugar translocation through the membrane (Ansari *et al.* 2017; Geada *et al.* 2017). A large amount of glucose ($C_6H_{12}O_6$) contained in RBE is a central compound of carbohydrate metabolism, and it is a type of aldehyde group-bearing sugar (Hong & Wang 2017). Therefore, a 1 g/L RBE concentration is recommended for the prevention of fungal growth and mass proliferation of *C. vulgaris*. The ability to mass-cultivate microalgae with a small amount of external carbon source can reduce the cost of external carbon sources, which is a good way to reduce the cost of microalga-derived biodiesel.

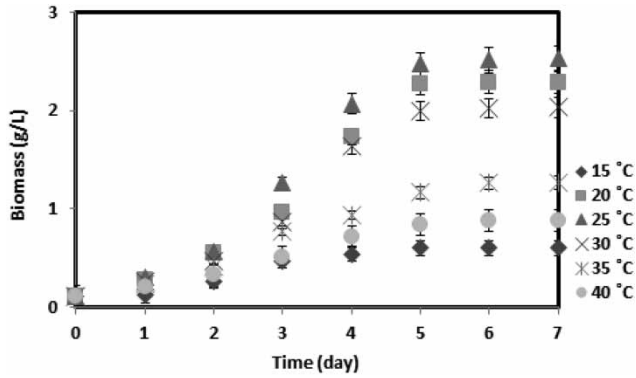

Figure 3 | Effect of temperature for biomass of *Chlorella vulgaris* (pH: 7–8, amount of rice bran extract: 1 g/L, and cultivation: 7 days, $n = 5$).

Effect of temperature

Besides oxygen concentration and pH, temperature is a mediating parameter that affects the growth of microalgae. Microalgae can grow at high and low temperatures depending on the species (Gupta *et al.* 2015). Temperature is an important factor in determining the growth rate, cell size, biochemical components, and nutrient requirements in microalgae growth; therefore, microalgae living at high and low temperatures differ in size and growth rate (Geada *et al.* 2017). In order to investigate the effect of temperature on the growth of *C. vulgaris* in this experiment, the optimum temperature condition was determined by fixing the RBE concentration to 1 g/L, pH 7–8, and incubation period to 7 days. The optimum temperature for microalgae growth was 20–30 °C and the highest growth rate was observed at 25 °C (Figure 3).

In general, the optimum temperature for the growth of freshwater microalgae is 25 ± 2 °C (Lee *et al.* 2015). Even if RBE was used as an external carbon source, the optimum temperature conditions for the growth of microalgae did not significantly change. If the temperature is too low (below 16 °C), the growth rate of microalgae slows down. When the temperature is too high (above 35 °C), the activity of microalgae enzymes is inhibited and photosynthesis activity is decreased. As a result, the growth protein synthesis in the microalgae is decreased and the microalgae growth is slowed down (Huang *et al.* 2017). Moreover, temperature also affects lipid accumulation as well as microalgae growth. Although the cell density is increased below the optimum temperature, the lipid content of the microalgae decreases sharply with increasing temperature (Wu *et al.* 2017). Temperature-related lipid accumulation mechanisms may refer to the enzyme instability associated with carbon fixation (ribulose bisphosphate carboxylase/oxygenase: RuBisCO) and lipid biosynthesis (Acetyl CoA carboxylase) (Hong *et al.* 2017). Therefore, microalgae are not able to regulate the temperature inside the cell, and it is therefore necessary to maintain the optimum temperature for mass culture.

Relationship between biomass and the consumption of glucose in RBE

The correlation between the increasing biomass and the consumption of glucose in RBE was investigated by fixing the pH at 7–8, the temperature at 25 ± 2 °C, and the RBE at 1 g/L through the above parameter optimization experiment. As the biomass increased, the amount of glucose in the RBE decreased (Figure 4(a)). The correlation (R^2) between the biomass and the consumption of glucose was determined to be 0.9751, indicating that the glucose contained in RBE directly affected the biomass increase. The biomass of *C. vulgaris* with RBE increased continuously up to 5 days, showing 2.52 g/L biomass, and no significant change was observed thereafter. The microalgae were cultivated in JM without RBE, showing 0.73 g/L biomass on the fifth day (Figure 4(b)). The growth of *C. vulgaris* using RBE was about four times greater than that of JM.

Organic carbon sources used under mixotrophic conditions affect the biomass productivity and lipid content of microalgae. These effects depend on the type of organic carbon source and the concentration of organic carbon sources. Also, even the same carbon source has different effects on species of microalgae (Geada *et al.* 2017). Among the organic carbon sources such as glucose, glycerol, xylose, rhamnoose, fructose, sucrose, and galactose, glucose is known to be the most effective carbon source of microalgae (Li *et al.* 2014). However, due to the high cost of glucose, some researchers have used products derived

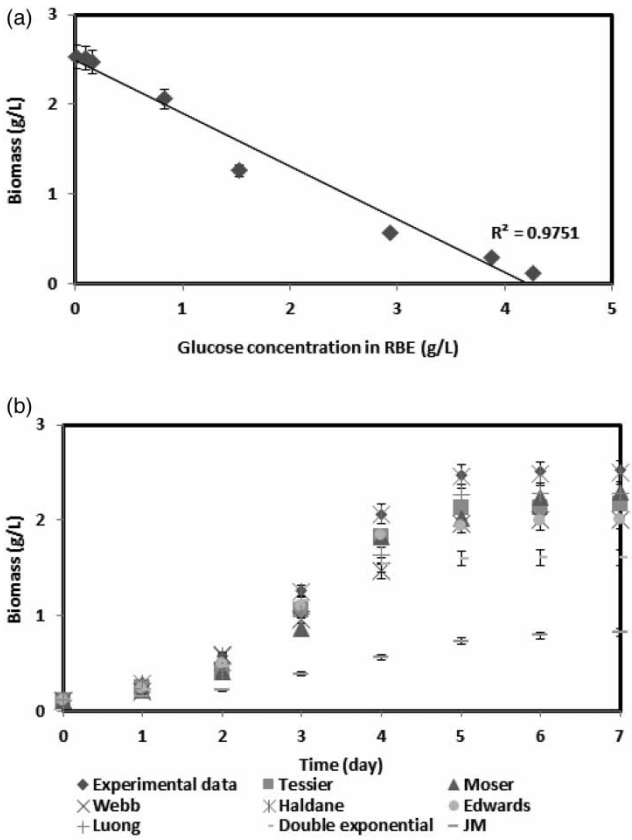

Figure 4 | (a) Relationship between biomass and glucose concentration in rice bran extract (RBE) (b) Biomass productivity with rice bran extract (RBE) and Jaworski's medium (JM) (pH: 7–8, amount of rice bran extract: 1 g/L, and temperature: 25 °C).

from the hydrolysis of cellulose as an alternative carbon source (Gupta *et al.* 2016a). Other researchers have reported that biomass and lipid productivity can be improved with low-cost biomaterial carbon sources such as sugar cane and yams (Choi & Lee 2015a).

The RBE used in the experiment is an eco-friendly agricultural waste. Because of the high glucose content in RBE, organic carbon source can be substituted for microbial biomass growth and lipid content at a low cost. However, when a large amount of an organic carbon source is added, the medium tends to become contaminated by fungi and other bacteria. Therefore, the use of organic carbon sources should be optimized when microalgae are grown under mixotrophic conditions. RBE is a very inexpensive and environmentally friendly discarded agricultural by-product. It is also significant as it represents a good example of resource recycling.

Kinetic models

Various models of microbial growth and biodegradation kinetics have been developed and proposed by many researchers. Using the kinetic model, we can explain the dynamics of substrate inhibition and growth, and analyze the effects of carbon source concentration, pH, and temperature on microalgae growth in order to reduce the time required for designing optimal conditions for mass culture. The effects of RBE on the growth of *C. vulgaris* were analyzed using various growth models including the Moser, Tessier, Webb, Haldane, Edwards, Double exponential, and Luong models. Each of these models is a modified Mond equation, and the parameters are upgraded by the empirical equation according to various conditions to modify and supplement the Mond model (Gokulakrishnan & Gummadi 2006). In other words, the Luong model describes the substrate inhibition kinetics of cell growth with a single restriction substrate (Luong 1987), the Haldane equation has been widely used to explain substrate inhibition dynamics and biodegradation of inhibitory substrates (Zhan *et al.* 2017), and the Moser equation has upgraded the Monod model to include parameters, incorporating the effects of the adoption of a fixed process by mutation (Halmi *et al.* 2014; Petrov & Ilkova 2016).

In this experiment, the effect of RBE concentration, pH, and temperature on *C. vulgaris* cultivation was examined by comparing and analyzing the experimental results using various control models. From the data analyzed using various models, we attempted to design a field plant for the mass production of *C. vulgaris* using RBE. The maximum growth rate according to the above optimization experiment was calculated using various control models. The results are shown in Table 2. The maximum growth rate ranged from 0.51 to 0.61 and the correlation coefficient R^2 ranged from 0.9927 to 0.9992 for all kinetic models. For the *C. vulgaris* growth experiment using RBE, the Luong model was most suitable as it has the highest correlation coefficient.

Table 3 compares the experimental results with previous studies in which microalgae were cultured in large quantities using various external carbon sources. Although relatively expensive glycerol or glucose is used as an external carbon source, it has been recently used for dairy wastewater, wine residue or waste water from tofu factories (Geada *et al.* 2017). However, cultivating microalgae in large quantities involves increased costs. In addition, dairy wastewater, wine residue, and wastewater from tofu plants have disadvantages because the environmental conditions suitable for microalgae growth such as pH, salinity, nutrient

Table 2 | Estimated parameters of various substrate inhibition models

Model	Equation	Parameters	R^2
Moser	$\mu = \mu_m S^n/(K_s + S^n)$	$n = 0.67$, $K_s = 0.93$, $\mu_m = 0.51$	0.9367
Tessier	$\mu = \mu_m[1 - \exp(-S/K_s)]$	$K_s = 1.36$, $\mu_m = 0.58$	0.9362
Webb	$\mu = \{\mu_m S[1 + (S/K_i)]\}/\{K_s + S + (S^2/K_i)\}$	$K_i = 1.14$, $K_s = 0.83$, $\mu_m = 0.58$	0.9582
Haldane	$\mu = \mu_m S/[(K_s + S) + (1 + S/K_i)]$	$K_i = 1.12$, $K_s = 1.07$, $\mu_m = 0.56$	0.9975
Edwards	$\mu = [\mu_m S/(K_s + S)]\ \exp^{(-S/Ki)}$	$K_i = 2.15$, $K_s = 0.99$, $\mu_m = 0.57$	0.9432
Double exponential	$\mu = \mu_m[\exp(-S/K_i) - \exp(-S/K_s)]$	$K_i = 2.55$, $K_s = 1.09$, $\mu_m = 0.57$	0.9672
Luong	$\mu = \mu_m S/(K_s + S)(1 - S/S_m)^n$	$S_m = 19.84$, $K_s = 0.99$, $\mu_m = 0.61$, $n = 2.41$	0.9992

Where μ (1/h) and μ_m (1/h) are the specific growth rate and maximum specific growth rate, respectively. S (mg/L), K_s (mg/L), and S_m (mg/L) are the limiting substrate concentration (RBE in this study), the Monod half saturation constant, and the maximum substrate inhibitory concentration at which no growth was observed, respectively. n is the constant which accounts for the relationship between μ and S. K_i (mg/L) is the substrate inhibition constant and its high value indicates that the culture is less sensitive to substrate inhibition. .

Table 3 | Comparison of mixotrophic performance of *Chlorella vulgaris* cultivated under different conditions according to literature and in this work

Microalgae strain	Carbon source	Dose of carbon source (g/L)	Lipid content (%)	Biomass (g/L)	Reference
Chlorella vulgaris	Cheese whey	2.2 (hydrolyzed)	32.0	2.6	Salati *et al.* (2017)
Chlorella vulgaris	Sodium acetate	5	42.5	1.2	Azizi *et al.* (2018)
	Sodium bicarbonate	5	26.3	1.3	Azizi *et al.* (2018)
Chlorella sp.	Sucrose	1	35.5	0.5	Lin & Wu (2015)
Chlorella sorokiniana	Na-Acetate	5	20.0	1.5	Mondal *et al.* (2017)
	Fructose	5	26.1	1.3	Mondal *et al.* (2017)
	Molasses	5	30.6	1.6	Mondal *et al.* (2017)
Chlorella vulgaris	Glucose	2	21.0	1.7	Fu *et al.* (2017)
Chlorella sp.	Glycerol	5	17.8	1.7	Ma *et al.* (2016)
Chlorella vulgaris	Rice bran extract	1	33.6	2.5	This study

concentration, and temperature must be controlled. However, large-scale microalgae cultivation using RBE is very economical because it is very simple to apply and waste is recycled because it can be mass cultivated using general conditions without the need to specially control the condition of the parameters. Compared with the previous studies, the production of *C. vulgaris* using RBE showed high biomass and high lipid content.

Lipid content and composition of total fatty acids

The lipid content of microalgae includes 30–60% of dry weight depending on the species. Also, the photosynthetic efficiency and lipid product potential of microalgae are higher than for land crops (Lin & Wu 2015). The lipid content of microalgae is a source of biofuel, a neutral compound that is stored predominantly in microalgae under stressful conditions such as strong and high amounts of light or malnutrition (Li *et al.* 2014; Mondal *et al.* 2017). That is, under environmental stress conditions such as nutrient starvation and strong light, microalgae rapidly stop dividing and lipids are accumulated in the cells (Fu *et al.* 2017). Table 4 compares the lipid content of *C. vulgaris* grown on RBE with *C. vulgaris* grown on JM. *C. vulgaris* grown on RBE showed lipid content of 33.6%, while *C. vulgaris* cultivated on JM showed lipid content of about 10.7%, indicating that *C. vulgaris* grown on RBE had about 3.2 times more lipid content than that cultivated on JM. This is probably because RBE contains a large amount of sugar, including glucose, which is believed to store more lipids than *C. vulgaris* grown on JM. When microalgae are cultured in mixotrophic conditions, they can produce 4.98 times more biomass than those cultured in autotrophic conditions and 2.28 times higher biomass than those cultured in heterotrophic conditions (Gupta *et al.* 2015). In addition, while the lipid content is 10–14.5% when grown in autotrophic conditions, this increases to 20–25% and 25–35% in the heterotrophic and mixotrophic conditions, respectively

Table 4 | Lipid content in dry mass of investigated algae species and composition of total fatty acid profiles of *Chlorella vulgaris* oil

	Lipid content in dry biomass of algae* (%)	Composition of total fatty acids (%)	
		saturated	unsaturated
JM	10.7 ± 1.3	37.0 ± 1.2	63.0 ± 2.5
RBE	33.6 ± 3.6	34.0 ± 1.2	66.1 ± 2.6

Data from 7-day cell growth of medium, pH 7–8, amount of RBE: 1 g/L and temperature: 25 °C.
JM: Jaworski's medium, RBE: Rice bran extract.

(Li *et al.* 2014; Gupta *et al.* 2016c). In particular, when the quantity of glucose increased, which improves the photosynthesis and absorption capacity of microalgae, the lipid content in the microalgae cells increased by more than 30% (Geada *et al.* 2017).

The microalgae are mostly single cells. Depending on the species, the lipid content and the chain length of the fatty acids contained may vary from C10 to C24 (Choi & Lee 2014). The contents of saturated and unsaturated fatty acids are important for using oil or vegetable oil produced from microalgae as biodiesel. The high content of saturated fatty acids means that biodiesel also has a high degree of saturation and can easily cause problems when using biodiesel higher than gasoline in winter due to its hardness (Choi & Lee 2019). Therefore, the content of saturated fatty acids is important when using vegetable or animal oils as biodiesel. The total saturated fatty acid content of *C. vulgaris* in JM was 37.0%, which was about 3% higher than that of *C. vulgaris* in RBE. That is, the content of saturated fatty acid decreased by 3% and the content of unsaturated fatty acid increased by 3% in *C. vulgaris* grown on RBE compared with *C. vulgaris* grown on JM. In general, saturated fatty acids are very stable and polyunsaturated fatty acids are faster to oxidize than monounsaturated fatty acids (Choi & Lee 2015b). As the level of unsaturation increases, oxidation is considered to be about 10 times faster (Choi & Lee 2014). Thus, the higher the content of unsaturated fatty acids in the biodiesel component, the faster the automatic oxidation proceeds (Li *et al.* 2014).

Various carbon sources such as sodium acetate, fructose, glucose, glycerol, sucrose, and acetate have been successfully applied to increase the growth rate and lipid content of microalgae (Geada *et al.* 2017). However, these carbon sources incur additional cost. The commercial prices of glucose, glycerol, and acetate are currently 0.5–0.8, 0.6–0.7 and 0.9–0.94 USD/kg, respectively (Choi & Yu 2015; Gupta *et al.* 2016a). The RBE used in this study is very significant in terms of resource circulation because it is a recycled waste product, it is simple to process, inexpensive, and is very effective for increasing microalgae growth rate and lipid content. In addition, economic value can be created by producing high-value useful substances such as health supplements, natural pigments, and medicinal materials from biomass obtained through the mass culture of microalgae. Moreover, the large-scale cultivation of microalgae can contribute to the resolution of global environmental problems such as the reduction of atmospheric toxin concentrations through biochemical CO_2 fixation. In other words, microalgal biotechnology is a promising future industry that can promote the development of environmental industry along with the activation of biotechnology industry.

CONCLUSIONS

In order to investigate the effect of RBE on *C. vulgaris* cultivation, the growth rate of microalgae was analyzed using various growth control models according to the pH, temperature, and RBE amount, which are the main parameters of microalgal growth. In addition, the biomass grown on RBE was harvested and compared with *C. vulgaris* grown in JM in terms of the amount of lipid and lipid components. From the result, *C. vulgaris* showed the highest growth rate at pH 7–8, temperature of 25–30 °C, and RBE concentration of 1 g/L; also, four times more biomass was able to be harvested than that in the JM. In the analysis using various growth model analyses, the Luong model was the most suitable empirical formula for the mass culture experiment of *C. vulgaris* using RBE, with the highest correlation coefficient. The lipid contents of *C. vulgaris* grown in RBE were about three times higher than those of *C. vulgaris* grown in JM. In addition, the content of saturated fatty acids was about 3% less than that of *C. vulgaris* grown on JM. According to the above results, when *C. vulgaris* is cultured using RBE, high amounts of biomass and high lipid content can be obtained with a small amount of RBE. RBE is a discarded waste and has a high glucose content; it can thus be replaced by an organic carbon source to increase microbial biomass growth and lipid content at low cost.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no conflicts of interest.

STATEMENT OF INFORMED CONSENT, HUMAN/ ANIMAL RIGHTS

No conflicts, informed consent, human or animal rights applicable.

DECLARATION OF AUTHOR CONTRIBUTIONS

All authors whose names are listed in this manuscript certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, data, collection, analysis, writing, and revision of manuscript.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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