รายงานวิจัยฉบับสมบูรณ์

โครงการ Mass Production and Evaluation of Biochemical Composition of the High Carotenoid Photosynthetic Bacteria, *Rhodopseudomonas faecalis* as Diet and Probiotic for Fairy Shrimp of Thailand

(ทุนส่งเสริมนักวิจัยรุ่นใหม่)

โดย

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ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์
มหาวิทยาลัยขอนแก่น

กรกฎาคม 2558-มิถุนายน 2560

สัญญาเลขที่ TRG5880004

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย และ สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

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ชื่อโครงการ: Mass Production and Evaluation of Biochemical Composition of the High Carotenoid Photosynthetic Bacteria, *Rhodopseudomonas faecalis* as Diet and Probiotic for Fairy Shrimp of Thailand

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บทคัดย่อ: เมื่อไม่นานมานี้มีการพบว่าแบคทีเรียสังเคราะห์แสง *Rhodopseudomonas faecalis* PA2 เป็นแบคทีเรียที่สามารถผลิตแคโรทีนอยด์ได้และมีอัตราการผลิตชีวมวลสูง แต่ยังไม่เคยมีการนำมาเพาะเลี้ยงในสับสเตรทที่มีราคาถูก ในงานวิจัยนี้จึงได้นำ *Rps. faecalis* PA2 มาเลี้ยงในน้ำเสียชุมชนและพบว่าความเข้มแสงและอัตราการกวนที่เหมาะสมมีค่าเท่ากับ 4,000 ลักซ์ และ 150 รอบต่อนาที ตามลำดับ จากการขยายขนาดการผลิตในถังปฏิกรณ์ชีวภาพแบบใช้แสงพบว่า มีค่าอัตราการเจริญจำเพาะเท่ากับ 1.61 ต่อวัน โดยมีการผลิตชีวมวลสูงสุดเท่ากับ 33.9 กรัม/ลิตร มีค่า carotenoid yield, carotenoid production rate และ carotenoid productivity เท่ากับ 7.2 มก./กรัม, 74.3 มก./ลิตร/วัน และ 40.9 มก./ลิตร/วัน ตามลำดับ ปริมาณสารอาหารในเซลล์ที่เลี้ยงในน้ำเสียชุมชนเมื่อผ่านการทำแห้งแบบแช่เยือกแข็ง พบว่ามีโปรตีน 64.8 เปอร์เซนต์ และไขมัน 10.6 เปอร์เซนต์ มีปริมาณกรดอะมิโนจำเป็นคิดเป็น 72.6 เปอร์เซนต์ ของปริมาณโปรตีนทั้งหมด มีปริมาณกรดไขมันไม่อิ่มตัวสูงกว่ากรดไขมันอิ่มตัวที่ประกอบไปด้วยกรดไขมันไม่อิ่มตัวเชิงซ้อนและกรดไขมันที่จำเป็น ได้แก่ โอเมก้า 3 และโอเมก้า 6 โดยเฉพาะอย่างยิ่งกรดอัลฟ่าไลโนเลนิค (18:3, *n*-3) ซึ่งแสดงให้เห็นว่าสามารถใช้เป็นอาหารสัตว์ได้ดี

การใช้แบคทีเรียสังเคราะห์แสงเพื่อเป็นอาหารไรน้ำนางฟ้ายังไม่เคยมีการรายงานมาก่อนเมื่อเปรียบเทียบกับสาหร่ายขนาดเล็กและยีสต์ ดังนั้นในงานวิจัยนี้จึงศึกษาผลของสาหร่ายขนาดเล็ก ยีสต์และแบคทีเรียสังเคราะห์แสงต่ออัตราการรอดชีวิต การเจริญ และคุณภาพน้ำที่ใช้ในการเพาะเลี้ยงไรน้ำนางฟ้าสิรินธร *Streptocephalus sirindhornae* พบว่าอัตราการรอดชีวิตของนอเพลียสไรน้ำนางฟ้าที่เลี้ยงด้วยสาหร่าย *Chlorella vulgaris* และแบคทีเรียสังเคราะห์แสง *Rps. faecalis* มีค่าสูงกว่า 80 เปอร์เซนต์ ในขณะที่การเลี้ยงด้วยยีสต์ *Saccharomyces cerevisiae* มีการรอดชีวิตเพียง 4.4 เปอร์เซนต์ หลังจากการเพาะเลี้ยงเป็นเวลา 30 วัน ไรน้ำนางฟ้าตัวอ่อนและตัวเต็มวัยที่เลี้ยงด้วย *Rps. faecalis* จะมีอัตราการรอด

ชีวิต (46.7 เปอร์เซนต์) และอัตราการเจริญ (0.47 มม./วัน) สูงสุด ความเข้มข้นของแอมโมเนีย ไนไตรท์ และไนเตรทในน้ำที่ใช้เพาะเลี้ยง (วัดทุก 3 วัน) เมื่อเลี้ยงด้วย *Rps. faecalis* จะมีค่าต่ำสุดเมื่อเทียบกับการเลี้ยงด้วยจุลินทรีย์ชนิดอื่น การเลี้ยงด้วย *S. cerevisiae* จะทำให้มีความเข้มข้นของแอมโมเนียสูงสุด ปริมาณออกซิเจนที่ละลายต่ำสุด และมีความขุ่นที่มากเกินซึ่งการเปลี่ยนน้ำเพาะเลี้ยงบ่อย ๆ ไม่สามารถแก้ปัญหานี้ได้ จึงส่งผลให้มีอัตราการรอดชีวิตต่ำ (10 เปอร์เซนต์) ผลการทดลองนี้ชี้ให้เห็นว่า *Rps. faecalis* สามารถใช้เป็นอาหารไรน้ำนางฟ้า และช่วยกำจัดของเสียไนโตรเจนได้ และงานวิจัยนี้ยังได้แสดงให้เห็นถึงผลเสียของการใช้ยีสต์เป็นอาหารในไรน้ำนางฟ้าอีกด้วย

คำหลัก: แบคทีเรียสังเคราะห์แสง น้ำเสียชุมชน ไรน้ำนางฟ้า *Rhodopseudomonas faecalis*

Project Code: TRG5880004

Project Title: Mass Production and Evaluation of Biochemical Composition of the High Carotenoid Photosynthetic Bacteria, *Rhodopseudomonas faecalis* as Diet and Probiotic for Fairy Shrimp of Thailand

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Project Period: July 2015-June 2017

Abstract: Photosynthetic bacterium *Rhodopseudomonas faecalis* PA2 was recently proposed as a new carotenoid producer with relatively high biomass production but the mass production in cheap substrate remains unclear. In this study, *Rps. faecalis* PA2 was cultivated in domestic wastewater. The optimum light intensity and agitation speed were 4,000 lux and 150 rpm, respectively. Mass production in the photo-bioreactor showed that specific growth rate was 1.61 /day with the maximum biomass production of 33.9 g/L. Carotenoid yield, carotenoid production rate and carotenoid productivity were found to be 7.2 mg/g, 74.3 mg/L/day and 40.9 mg/L/day, respectively. The nutritional profile of the freeze dried bacterial biomass obtained from domestic wastewater contained 64.8% protein and 10.6% lipid. The essential amino acid (EAA) accounted for approximately 72.6% of the whole protein content. The content of unsaturated fatty acid was higher than saturated fatty acid consisting of polyunsaturated fatty acid (PUFA) and essential fatty acid including omega-3 and omega-6, particularly, alpha-linolenic acid (18:3, *n*-3), indicating to be used as the good feedstuff.

Compared to microalgae and yeast, utilization of photosynthetic bacteria as fairy shrimp's diet has not been reported in the literature. In this study, the effects of microalgae, yeast and photosynthetic bacteria as sole diet on survival, growth performance and water quality in fairy shrimp *Streptocephalus sirindhornae* Sanoamuang, Murugan, Weekers and Dumont, 2000 were first investigated. Survival of the larvae fed with algae *Chlorella vulgaris* and photosynthetic bacterium *Rhodopseudomonas faecalis* were higher than 80% while those fed with yeast

Saccharomyces cerevisiae was 4.4%. After 30 days of cultivation, sub-adult and adult fairy shrimp fed with *Rps. faecalis* showed the highest survival (46.7%) and growth rate (0.47 mm/day). Ammonia, nitrate and nitrite concentrations of the rearing water (measured 3 days interval) treated with *Rps. faecalis* were the lowest compared to the other microbes. The highest ammonia concentration, the lowest dissolved oxygen and excessive turbidity were found in the water treated with *S. cerevisiae* but the frequent water replacement could not overcome this incidence that resulted in the low survival rate (10%). The results indicate that utilization of *Rps. faecalis* as fairy shrimp's diet and nitrogen wastes removal are feasible and the disadvantages of yeast in fairy shrimp culture are proposed in this study.

Keywords: photosynthetic bacteria, domestic wastewater, fairy shrimp, *Rhodopseudomonas faecalis*

กิตติกรรมประกาศ

ผู้วิจัยขอขอบพระคุณ

สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) และมหาวิทยาลัยขอนแก่น ที่ได้ให้โอกาสผู้วิจัยได้รับทุนส่งเสริมนักวิจัยรุ่นใหม่ในการทำงานวิจัยครั้งนี้

ศาสตราจารย์ ดร.ละออศรี เสนาะเมือง ที่ตอบรับเป็นนักวิจัยที่ปรึกษาให้กับโครงการนี้ และยังเคยเป็นอาจารย์ที่ปรึกษาวิทยานิพนธ์ให้กับผู้วิจัย

คณะผู้ประเมิน (reviewer) ของวารสารวิชาการต่าง ๆ ที่ได้ให้คำแนะนำ ตลอดทั้งปรับปรุงต้นฉบับของบทความที่ส่งไปเพื่อตีพิมพ์ในวารสารนั้น ๆ

นักศึกษาและเจ้าหน้าที่ฝ่ายสนับสนุน ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น ที่ได้ร่วมศึกษาวิจัยและช่วยเหลือโครงการวิจัยในครั้งนี้

ชีวาพัฒน์ แซ่จึง

Chapter 1

Executive Summary

This study indicates the mass production of photosynthetic bacterium *Rhodopseudomonas faecalis* PA2 in the undiluted domestic wastewater without nutrient supplementation. The physical parameters including light intensity of 4,000 lux and agitation speed of 150 rpm were optimal for cultivation. The biomass obtained from the mass production showed the high carotenoid, protein and lipid contents with essential amino acids and essential fatty acids which meet the requirements for animal diets. Based on the results, *Rps. faecalis* PA2 grown in domestic wastewater is feasible to be used as a good feedstuff for animal feed with price competitiveness. Therefore, this strain was used as a diet in the indigenous fairy shrimp *Streptocephalus sirindhornae* compared to microalgae and yeast. Survival and growth rate of fairy shrimp fed with *Rps. faecalis* were higher than those fed with *Chlorella vulgaris* and *Saccharomyces cerevisiae*. Moreover, ammonia, nitrite and nitrate concentrations of the rearing water treated with photosynthetic bacterium were the lowest. The results indicate that utilization of *Rps. faecalis* as fairy shrimp's diet and nitrogen wastes removal are feasible and this strain should be recommended in fairy shrimp culture of Thailand.

Chapter 2

Main Results

Objectives

1. To study the mass production of photosynthetic bacteria *Rhodopseudomonas faecalis* grown in domestic wastewater

2. To provide the biochemical profile of *Rps. faecalis* grown in domestic wastewater

3. To investigate the efficiency of *Rps. faecalis* on growth performance, survival rate and duration of maturation in fairy shrimp *Streptocephalus sirindhornae* compared to the known microbial live food, *Chlorella vulgaris* and *Saccharomyces cerevisiae*.

Materials and Methods

1. Photosynthetic bacteria and wastewater analysis

Photosynthetic bacterium *Rps. faecalis* PA2, isolated from wastewater treatment pond, was used in this study. The strain was grown in the modified glutamate-malate (GM) medium for 72 h under anoxygenic condition at ambient temperature (26-30^{o}C) (Saejung & Apaiwong).

Undiluted domestic wastewater, collected from wastewater treatment pond located in Khon Kaen University Thailand, was used as the sole substrate in the experiments. Prior to use, the wastewater was filtered through the filter nylon with a size of 60 μm to separate the large particles and sediment. After filtration, it was sterilized at 121^{o}C for 30 min. To determine the characteristics of sterile wastewater, closed reflux method (standard method part 5220 C), Kjedahl method, stannous chloride method, standard method part 2540 D and drying at 105^{o}C were used to analyzed the chemical oxygen demand (COD), total Kjedahl nitrogen (TKN), total phosphate (TP), total suspended solids (TSS) and total solids (TS), respectively.

2. Optimization studies in domestic wastewater

The experiments were carried out in a 500-mL screw-capped production bottle completely filled with sterile wastewater to generate anoxygenic condition. The pH was adjusted to 7.0. The parameters of inoculum were as follows: inoculant age of 72 h, OD_{660} of 0.5 and inoculant volume of 10% (v/v). To optimize the important physical parameters, the effect of light intensity (2,000, 3,000, 4,000 and 5,000 lux) and agitation speed (static condition, 150, 300 and 600 rpm) were investigated. Nitrogen gas was flushed into the production bottles to keep anoxygenic condition. The experiment was done in triplicate.

3. Mass production of *Rps. faecalis* PA2 in domestic wastewater

Batch cultivation was conducted in a 5 L photo-bioreactor leaving a small air space between culture broth and the lid of bioreactor in order to maintain anoxygenic condition. The reactor was sterilized at 121 ^{o}C for 30 min before use. Ilumination was provided by an external light source. Inoculant age and volume were 72 h and 10%, respectively. Anoxygenic condition was created by flushing nitrogen gas (99% purity) into the photo-bioreactor as indicated in Fig. 1. The optimum light intensity and agitation speed from the previous experiments were used in the batch cultivation.

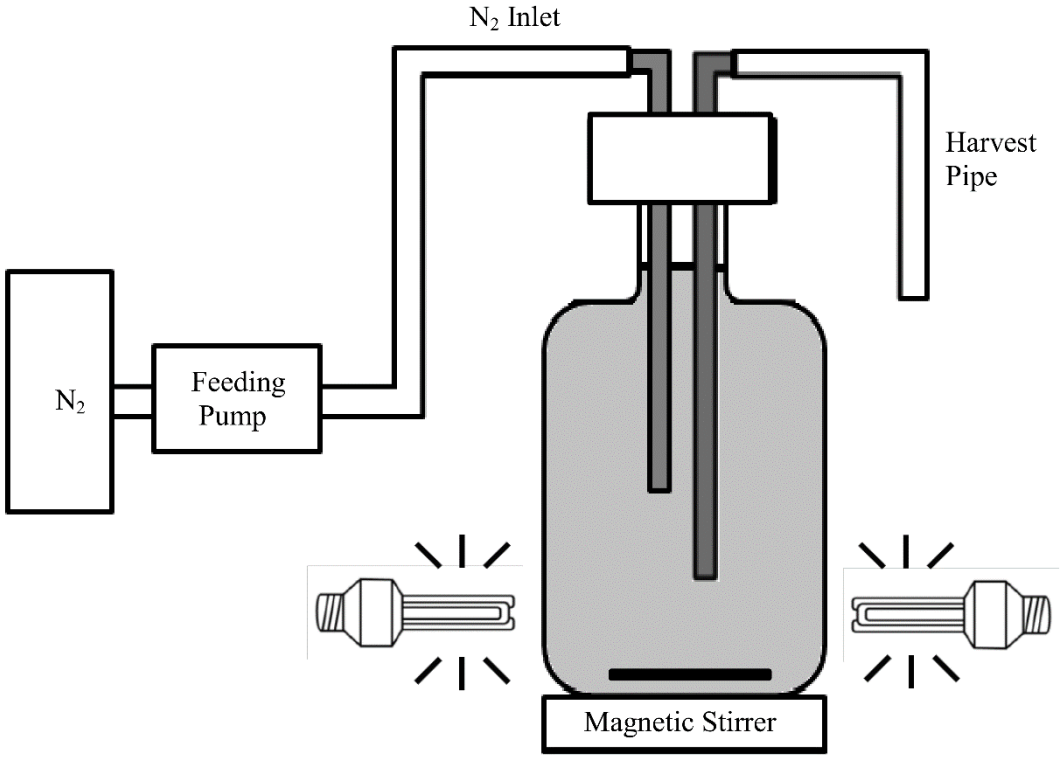

Fig. 1 Photo-bioreactor

4. Biochemical analysis

Bacterial cell grown in domestic wastewater was harvested by centrifugation and washed cell with sterile 0.85% (w/v) saline solution. The cell pellet was freeze dried using a freeze dryer (Freezone 2.5L; LABCONCO, Kansas City, United States). Protein and lipid of the freeze dried biomass were analyzed using Kjeldahl method and soxhlet extraction, respectively (AOAC, 1995). Amino acid composition of the freeze dried sample was determined by using an in-house method based on AOAC (2000) and detected by gas chromatography-mass spectrometry (GC-MS). The fatty acid methyl esters (FAMEs) were quantitatively measured by capillary gas chromatography (GC) based on (AOAC, 2005).

5. Analytical methods

The dry weight of bacterial biomass was used to determine the growth rate. Bacterial cell was harvested by centrifugation the culture broth at 6,000 rpm 4°C for 20 min (Himac CR20B2; Hitachi, Tokyo, Japan) and washed twice with 0.85% (w/v) saline solution. Carotenoid and bacteriochlorophyll were extracted using methanol-acetone (2:3 v/v) until the cell was colorless as previously described by Hirayama (1968).

6. Microorganism cultivation used for fairy shrimp culture

Rps. faecalis PA2 was grown in glutamate-malate medium under static anoxygenic-light condition and incubated at room temperature. The microalgae *C. vulgaris* was grown in BG-11 medium under oxygenic-light condition in the shaker at room temperature. The yeast *S. cerevisiae* was grown in YM medium under oxygenic condition in the shaker at room temperature. Due to liquid form and small size, these microorganisms could be fed to the fairy shrimp directly without mixing with any particles.

7. Fairy shrimp and cyst hatching

The indigenous fairy shrimp, *S. sirindhornae*, was used in this study. Reverse osmosis water pH 6.5-8.0 was used to rear the fairy shrimp. Cyst of the fairy shrimp were submerged in the glass container containing 1 L of water under static room condition for 24 h at water temperatures between 24 and 26°C. These are common temperatures when fairy shrimp occur during rainy season in Thailand. After hatching, the larvae were transferred to the tested container containing 2 L of water.

8. The effects of different microorganisms as diet on survival and water quality of the larvae fairy shrimp

Feeding was assigned once a day and started 1 day after hatching. *Rps. faecalis*, *C. vulgaris* and *S. cerevisiae* were used as the tested microorganisms. Each treatment with different microorganisms had 15 individuals with 6 replicates. The concentration of bacteria, algae and yeast was adjusted approximately 2.5 x 10^5 cell/mL. All the experiments were conducted under static room conditions at a visible light intensity of 1,140 lux. The larvae were reared for 3 days because the larvae of *S. sirindhornae* was commonly reared for 2-3 days in Thailand. Survival rate and water quality including

dissolved oxygen (DO), ammonia, nitrite and nitrate concentrations were measured at the end of the experiment. Survival rate was calculated according to equation 1 (Saejung et al., 2014).

$$\text{Survival rate} = \frac{(15 - N_x) \times 100}{15} \quad (1)$$

Where 15 is the number of larvae/adult fairy shrimp used in each experiment and N_x denotes the number of the dead larvae.

9. The effects of different microorganisms as diet on survival, growth performance and water quality of the sub-adult and adult fairy shrimp

Cyst of *S. sirindhornae* were hatched and reared in the glass container containing 1 L of water as previously described. The selected microorganism obtained from the larvae experiment was fed to the new hatched fairy shrimp for 5 days. *Rps. faecalis*, *C. vulgaris* and *S. cerevisiae* were fed to the fairy shrimp started on day 6 after hatching. Each treatment had 10 individuals (10 individuals/ 3 L of water) with 6 replicates. Feeding was assigned once a day. The concentration of the microbes fed to the sub-adult and adult stages was 0.5 x 10^6 cell/mL. The cultivation was conducted under static room condition at light intensity of 1,140 lux. During the cultivation, fecal particles were siphoned and reverse osmosis water was partially changed 3 days interval. The first 3 replicates were used to determine survival rate at the end of subsequent culture periods 3 days interval. Survival rate was calculated according to equation 1. The latter 3 replicates were used to investigate the growth rate. Growth performance was estimated by measuring body length along with their development 3 days interval. Measurement of individual was made from tip of the head to the posterior margin of telson using a vernier caliper (Dararat et al. 2011). Growth rate was determined as millimeter body length per day (mm/day) according to equation 2.

$$\text{Growth rate (mm/day)} = \frac{\text{Body length (mm)}}{\text{Cultivation time (day)}} \quad (2)$$

Additionally, the duration of maturation, when eggs were first present in the brood pouch of females and the full-grown antennae were formed on the males, was observed. The pH, DO, ammonia, nitrite and nitrate concentrations of the rearing water

were measured 3 days interval in the latter 3 replicates before changing the rearing water. The experiments were done for 30 days.

10. Analytical methods of rearing water

DO and pH were measured by DO meter (YSI model 550A, YSI Incorporated, USA) and pH-meter (PCTestr 35, Eutech Instruments Pte Ltd., Singapore), respectively. Ammonia was analyzed by Nesslerization method (APHA, AWWA & WEF 1992). Nitrite and nitrate concentrations were analyzed by spectrophotometric method (4500B) and ultraviolet spectrophotometric method, respectively (Armstrong, 1963; APHA, AWWA & WEF, 1980).

11. Statistical analysis

Survival rate and growth rate of fairy shrimp and water quality were analyzed using one-way analysis of variance (ANOVA). The Least Significant Difference (LSD) was employed to detect significant differences among treatments at the 0.05 significance level. All data analysis were carried out using the SPSS Version 17.0.

Results

1. Feasibility study of biomass and carotenoid production of *Rps. faecalis* PA2 in domestic wastewater

The characteristics of domestic wastewater is shown in Table 1. The feasibility study of the selected strain in wastewater was carried out in light intensity at 2,000 lux and pH 7.0. As shown in Fig. 2, it was feasible to use domestic wastewater as substrate for biomass and pigment production in *Rps. faecalis* PA2. The biomass production was increased quickly during the first 7 days without the lag phase, indicating that this strain could effectively utilize organic and inorganic compounds in the wastewater for growth. Carotenoid production was increased considerably at the end of the exponential to stationary growth phase because the pigments were secondary metabolites that were directly involved in photosynthesis and anti-oxidation rather than the normal growth of bacteria. From the observation of this strain grown in domestic wastewater, the final biomass production, carotenoid production and bacteriochlorophyll production were found to be 13.3 g/L, 162.2 mg/L and 696.3 mg/L, respectively. Compared to the results obtained from Saejung and Apaiwong (2015), biomass and carotenoid production of the strain PA2 grown in chemically synthetic medium were 40 g/L and 413 mg/L, respectively. To improve the productivity of *Rps. faecalis* PA2 grown in domestic wastewater, optimization of the culture condition is needed.

Table 1 The characteristics of domestic wastewater used in this study.

Parameters	**Value (mg/L)**
Chemical oxygen demand	4,500 ± 5.55
Total Kjedahl nitrogen	176 ± 2.58
Total phosphate	121 ± 0.79
Total suspended solids	6.5 ± 0.23
Total solids	125 ± 6.72

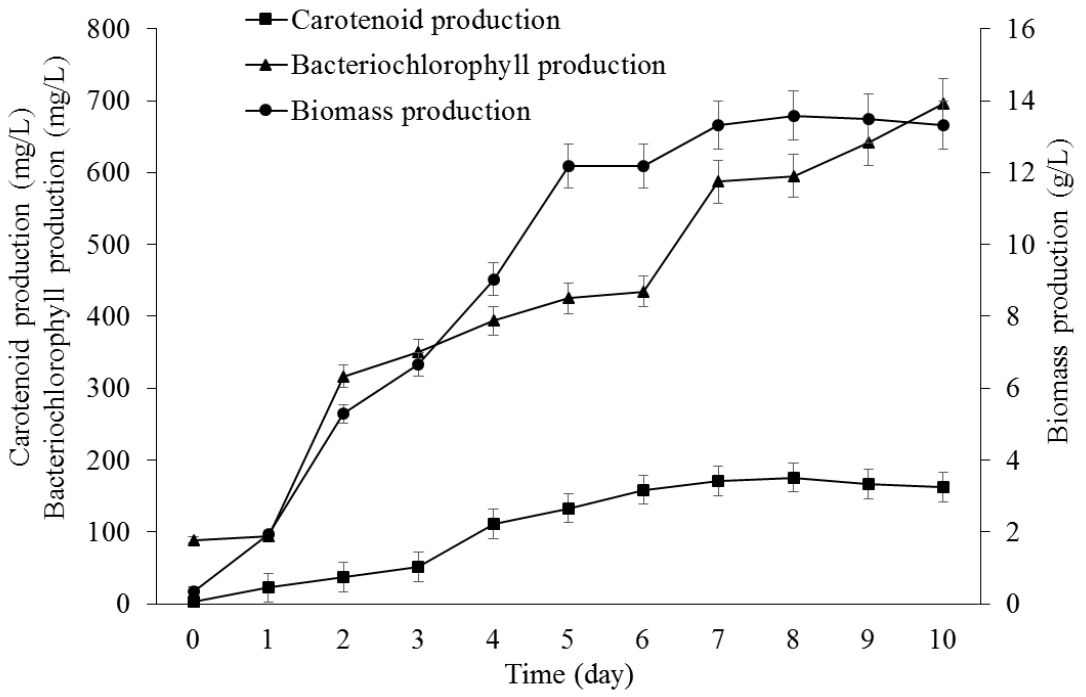

Fig. 2 Biomass, carotenoid and bacteriochlorophyll production of *Rhodopseudomonas faecalis* PA2 cultivated in domestic wastewater.

2. Effect of light intensity on biomass and carotenoid production of *Rps. faecalis* PA2 in domestic wastewater

As shown in Fig. 3A, specific growth rate was increased when light intensity was increased from 2,000 to 4,000 lux. The explanation is that the higher light intensity, the higher energy obtained *via* photosynthesis, resulting in the highest biomass productivity and biomass production (Figs. 3A and 3B). Results showed that light at 4,000 lux was the threshold in this strain, beyond which the biomass concentration was decreased.

Carotenoid production profile was similar to that of the biomass production. Carotenoid production was enhanced when light intensity was increased (Fig. 3C). Generally, carotenoids are secondary metabolites producing in stationary phase. In this study, carotenoid concentration was increased even in the late exponential phase. As shown in Fig. 3D, light intensity of 4,000 lux was the most appropriate because it gave the highest carotenoid productivity. Moreover, light at 4,000 lux was found to exhibit the highest carotenoid yield which referred to high carotenoid produced per gram cell, suggesting profitable production.

However, carotenoid production was decreased when the strain was exposed to light intensity beyond 4,000 lux. Strong light intensity can induce the excessive excitation in the photosynthetic apparatus. Exposure to excessive light intensity resulted in the generation of singlet oxygen. The formation of singlet oxygen leads to the loss of protein subunit (H, M and L) found in bacterial reaction center that leads to severely damage in photosynthetic organisms. (Li et al., 2014; Tandori et al., 2001). Therefore, light intensity beyond 4,000 lux was harmful to *Rps. faecalis* PA2 because it could induce the formation of dangerous oxygen species in the photochemical reaction centers.

Bacteriochlorophyll production was used to evaluate photosynthesis rate of phototrophic bacteria in the presence of light intensity. Likewise, bacteriochlorophyll production trend was similar to those of biomass and carotenoid production (Fig. 3E). Bacteriochlorophyll concentration was the highest when the strain was exposed to the light at 4,000 lux, whereas the production of bacteriochlorophyll was decreased above this light intensity.

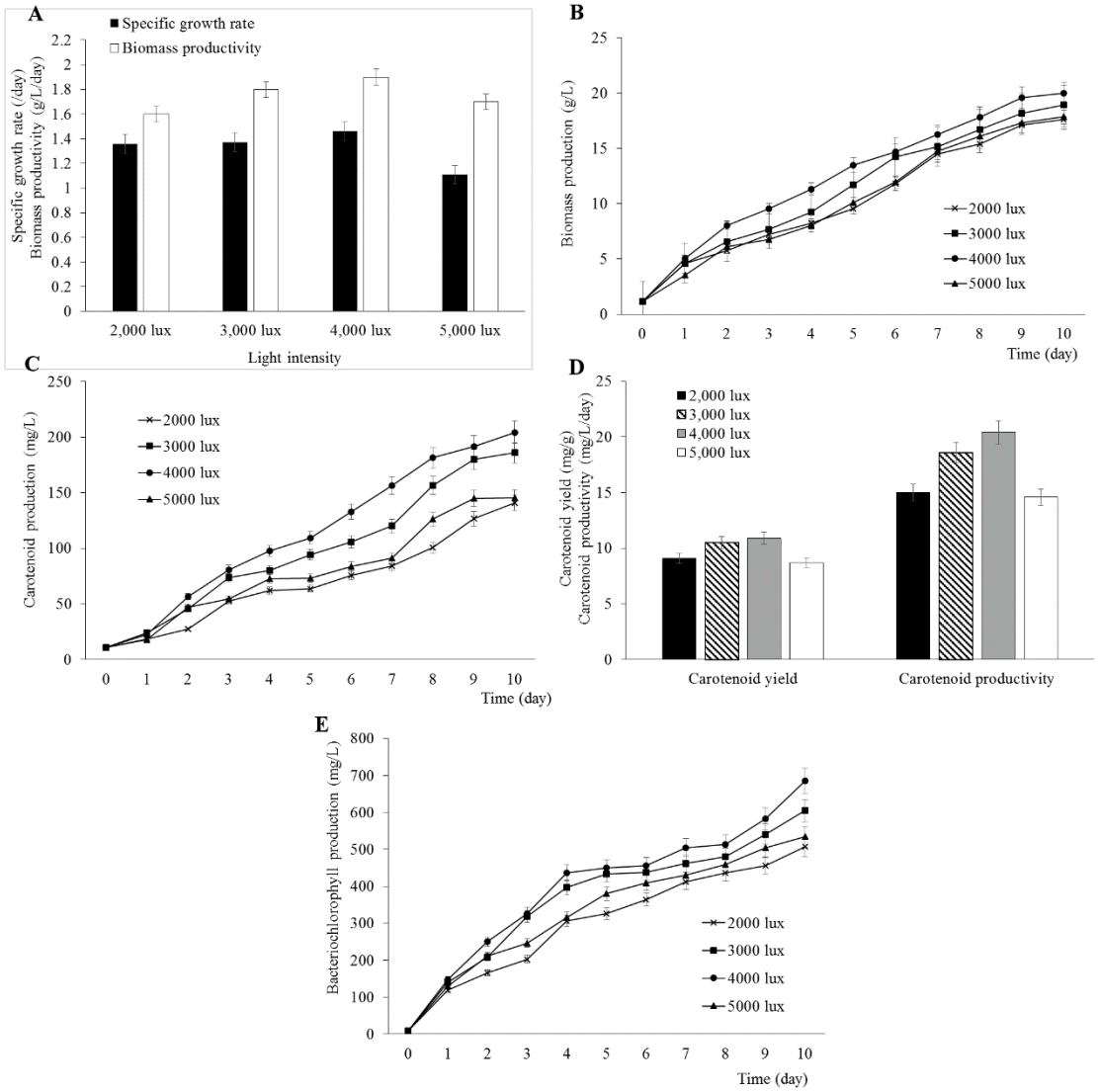

Fig. 3 Effect of light intensity on specific growth rate and biomass productivity (A), biomass production (B), carotenoid production (C), carotenoid yield and carotenoid productivity (D) and bacteriochlorophyll production (E) in *Rhodopseudomonas faecalis* PA2 cultivated in domestic wastewater.

3. Effect of agitation speed on biomass and carotenoid production of *Rps. faecalis* PA2 in domestic wastewater

Actually, this strain could grow well under static condition with high biomass concentration. However, the period of exponential phase was very long resulted in the slow growth and the duration to reach to stationary phase is delayed (Fig. 3B). Although the incubation time was terminated, bacterial growth was still in the exponential phase. These have an adverse effect on carotenoid production because carotenoids are

secondary metabolite and highly produce in the stationary phase. Furthermore, the delay in growth rate are time and cost consuming which is not satisfying in industrial production. In addition to the long cultivation time, the flocculation and the occurrence of bacterial cells attach themselves to side surfaces of the culture bottle to form adherent bacterial film, known as wall growth, were the problem. A thick layer formation caused the light transfer limitation to phototrophic bacteria. Therefore, agitation of the culture broth was studied to minimize the cultivation time and reduce the light transfer limitation.

As indicated in Fig. 4A, specific growth rate and biomass productivity were the highest under agitation speed at 150 rpm. Specifically, biomass productivity was increased under agitation compared with static condition. As shown in the results, the specific growth rate and biomass productivity obtained from agitation speed at 150 rpm were higher than those from agitation speed at 300 and 600 rpm. This was due to the occurrence of shear stress when bacterial cell was exposed to the high agitation speed. Bacterial growth under static condition showed the long exponential phase up to 9 days, whereas the growth of bacteria under agitation reached to a stationary phase within 4 days (Fig. 4B). This phenomenon was probably due to the effect of mixing which provided homogenous culture and increased light transfer. Additionally, agitation prevents bacterial clumps or biofilm formation on the side of the culture bottle as well as to avoid bacterial sedimentation on the bottom, improving the nutrient availability. Therefore, agitation speed at 150 rpm led to minimize the cultivation time and operating cost of biomass production.

As shown in Fig. 4C, carotenoid production was the highest when the strain was cultured under agitation speed of 150 rpm. The highest carotenoid content was obtained within 8 days which reduced the hydraulic retention time for carotenoid production resulted in the highest carotenoid productivity (Fig. 4D). Carotenoid yield was the highest under agitation speed of 150 rpm, whereas the lowest carotenoid yield was found in the static condition. Actually, carotenoid is highly produced in the presence of light and it is repressed in the dark and low light intensity. Probably, it could be assumed that the occurrence of bacterial biofilm caused the light transfer limitation on bacterial cell cultivated in static condition, thus, decreasing carotenoid biosynthesis. On the other hand, agitation is known to minimize concentration gradients of substrate and provide homogenous liquid culture that prevent surface attachment and aggregation, thus, increasing cell surface area for exposure to the light throughout the culture broth. When

the light transfer increased, carotenoid accumulation was enhanced. Although moderate agitation facilitated carotenoid production, excessive agitation speed could destroyed the cells and preventing proper replication. This phenomenon might support our results obtained from the experiment under agitation speed at 300 and 600 rpm.

Bacteriochlorophyll production under agitation speed of 150 rpm was not significantly different from that of static condition (Fig. 4E). Bacteriochlorophyll concentration was low under agitation speed of 300 and 600 rpm. It seems reasonable to assume that the low production of bacteriochlorophyll caused by shear stress under high agitation speed, resulting in reduced bacteriochlorophyll concentration.

According to the optimization studies, the optimum conditions, biomass production, carotenoid production, bacteriochlorophyll production, specific growth rate, carotenoid yield, carotenoid productivity and biomass productivity are summarized in Table 2.

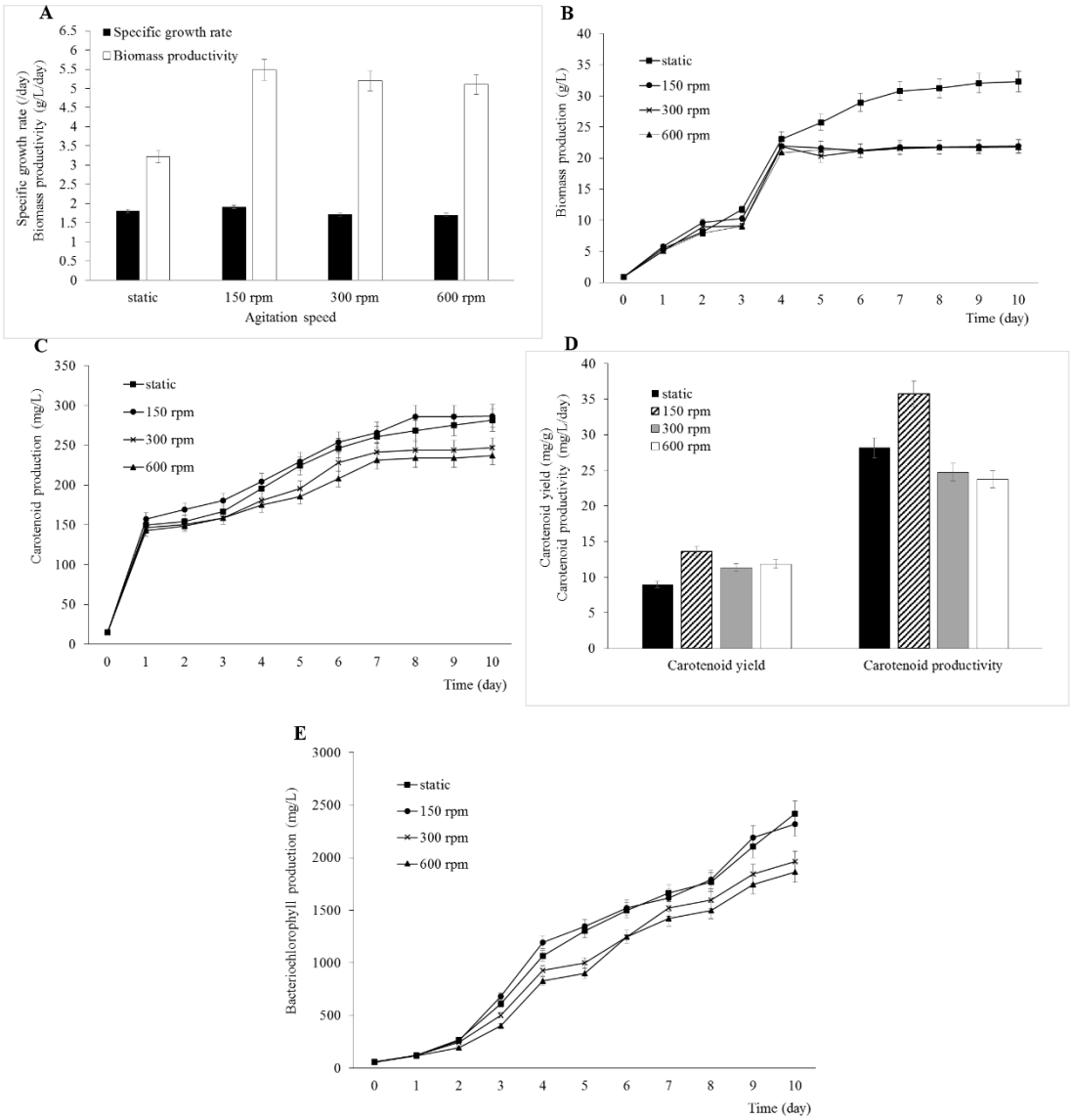

Fig. 4 Effect of agitation speed on specific growth rate and biomass productivity (A), biomass production (B), carotenoid production (C), carotenoid yield and carotenoid productivity (D) and bacteriochlorophyll production (E) in *Rhodopseudomonas faecalis* PA2 cultivated in domestic wastewater.

Table 2 The optimum conditions of *Rhodopseudomonas faecalis* PA2 grown in domestic wastewater obtained from the optimization studies.

Factors	Optimum value	Specific growth rate	Biomass production	Carotenoid production	Bacteriochlorophyll production	Carotenoid yield	Carotenoid productivity	Biomass productivity
Light intensity	4,000 lux	1.46	18.8	204.3	685.8	10.9	20.4[a]	1.9[a]
Agitation speed	150 rpm	1.91	21.1	286.8	2,319.5	13.6	35.7[b]	5.3[c]

[a], Calculated from 10 days.

[b], Calculated from 8 days.

[c], Calculated from 4 days.

4. Mass production of *Rps. faecalis* PA2 in domestic wastewater

The optimum conditions obtained in Table 2 were used to culture *Rps. faecalis* PA2 in a 5 L photo-bioreactor. As shown in Fig. 5A, the strain grew exponentially without the lag phase and the number of new cells created was limited after 4 days of cultivation. The kinetic parameters of the mass production are presented in Table 3. The specific growth rate was 15.7% lower in the mass production than in the agitation experiment. It could be assumed that the large cultivation vessel caused the light transfer limitation. However, the maximum biomass production and biomass productivity obtained from a photo-bioreactor were increased by 60.7 and 7.5%, respectively relative to the optimization studies. The cultivation time for the maximum carotenoid production presented in the optimization experiments was 8 days (Fig. 4C) while this occurred on day 6 in the mass production (Fig. 5B). Therefore, carotenoid productivity was 14.6% higher in the mass production than in the optimization studies. Biomass and carotenoid production rates were found to be 11.7 g/L/day and 74.3 mg/L/day, respectively (Table 3). Bacteriochlorophyll production was increased higher than that obtained from the optimization studies. The highest bacteriochlorophyll content of 2,320 mg/L was produced on day 10 in the agitation experiment (Fig. 4E) while the highest content was found to be 2,332 mg/L on day 8 in the mass production (Fig. 5C).

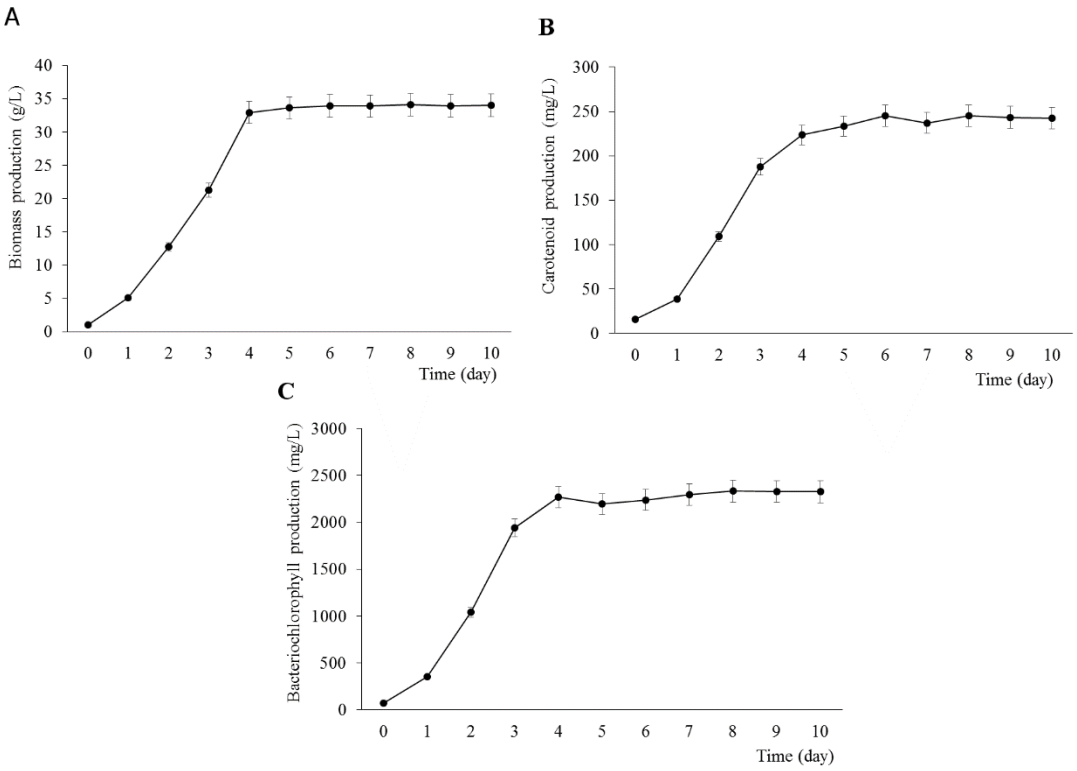

Fig. 5 Mass production of *Rhodopseudomonas faecalis* PA2 cultivated in domestic wastewater in a 5 L photo-bioreactor; biomass production (A), carotenoid production (B) and bacteriochlorophyll production (C).

Table 3 Kinetic parameters obtained from the batch cultivation of *Rhodopseudomonas faecalis* PA2 grown in domestic wastewater using a 5 L photo-bioreactor.

Parameters	**Value**	**Unit**
Specific growth rate	1.61	/day
Maximum carotenoid production	245.1	mg/L
Maximum biomass production	33.9	g/L
Carotenoid yield	7.2	mg/g
Carotenoid productivity	40.9[a]	mg/L/day
Biomass productivity	5.7[a]	g/L/day
Carotenoid production rate	74.3	mg/L/day
Biomass production rate	11.7	g/L/day

[a], Calculated from 6 days.

Obviously, the carotenoid productivity obtained from the GM medium was not significantly different from that obtained by cultivating in the domestic wastewater. Moreover, the biomass concentration presented in this study increased twice. This indicates domestic wastewater can be used for mass production of *Rps. faecalis* PA2.

5. Biochemical composition of *Rhodopseudomonas faecalis* PA2

Since photosynthetic bacterial cells contain protein, heat-labile and hormone-like growth substances, vitamin E and B complex, thus, they are widely used as feedstock in poultry and aquatic animals. Besides photosynthetic bacteria, yeast and algae are also utilized as feed supplement in aquaculture. Therefore, the biochemical composition of *Rps. faecalis* PA2 grown in domestic wastewater are compiled in Table 4 and compared with the food species of photosynthetic bacteria, yeast and algae. As indicated in Table 4, the freeze dried biomass of *Rps. faecalis* PA2 contained the high content of crude protein.

Table 4 Protein and lipid contents of *Rhodopseudomonas faecalis* PA2.

Biochemical composition	Content (%)
protein	64.8
lipid	10.6

The freeze dried biomass of *Rps. faecalis* PA2 was used to evaluate amino acid composition as presented in Table 5. The essential amino acid accounted for approximately 72.6% of the whole protein content.

Table 5 Amino acid composition of *Rhodopseudomonas faecalis* PA2 biomass grown in domestic wastewater and dietary amino acid requirement for penaeid shrimp.

Amino acid	***Rps. faecalis* PA2 (g/100 g dry cell)**
Histidine	4.39
Lysine	13.73
Phenylalanine	7.22
Leucine	6.84
Isoleucine	2.56
Tryptophan	1.32
Valine	2.36
Threonine	0.73
Methionine	0.45
Cysteine	0.38
Alanine	2.17
Glycine	1.13
Proline	1.19
Glutamic acid	2.61
Serine	0.47
Tyrosine	5.24
Aspartic acid	1.76

Fatty acid composition of *Rps. faecalis* PA2 was determined as shown in Table 6. Obviously, the content of unsaturated fatty acid was higher than saturated fatty acid, indicating the good feedstuff. Additionally, bacterial biomass contained polyunsaturated fatty acid (PUFA) and essential fatty acid including omega-3 and omega-6 fatty acids, particularly, alpha-linolenic acid (18:3, *n*-3) which is necessary for shrimp growth.

Table 6 Fatty acid composition of *Rhodopseudomonas faecalis* PA2 biomass grown in domestic wastewater.

Fatty Acid	Content (g/100 g dry cell)
Saturated fatty acid	1.811
Lauric acid (12:0)	0.031
Myristic acid (14:0)	0.087
Pentadecanoic acid (15:0)	0.007
Palmitic acid (16:0)	2.225
Heptadecanoic acid (17:0)	0.066
Stearic acid (18:0)	0.395
Unsaturated fatty acid	7.702
Palmitoleic acid (16:1, *n*-7)	0.331
cis-9-Oleic acid (18:1, *n*-9)	7.046
alpha-Linolenic acid (18:3, *n*-3)	0.032
cis-8,11,14-Eicosatrienoic acid (20:3, *n*-6)	0.292
Trans fatty acid	0.098
Trans-9-Elaidic acid (18:1, *n*-9t)	0.098

6. Survival and water quality of the larvae

As shown in Fig. 6, survival rate was significantly influenced by the type of feed. Survival rate of the larvae fed with *Rps. faecalis* and *C. vulgaris* was not significantly different ($p > 0.05$). The corresponding value were 82.2 and 86.7% when *Rps. faecalis* and *C. vulgaris* were used as the feed, whereas the treatment fed with *S. cerevisiae* showed only 4.4% survival ($p < 0.05$). As shown in the results, the use of *S. cerevisiae* as larvae feed was not successful. In order to conduct the subsequent experiments, *C. vulgaris* was used to rear the larvae for 5 days before feeding with different microorganisms in the following studies.

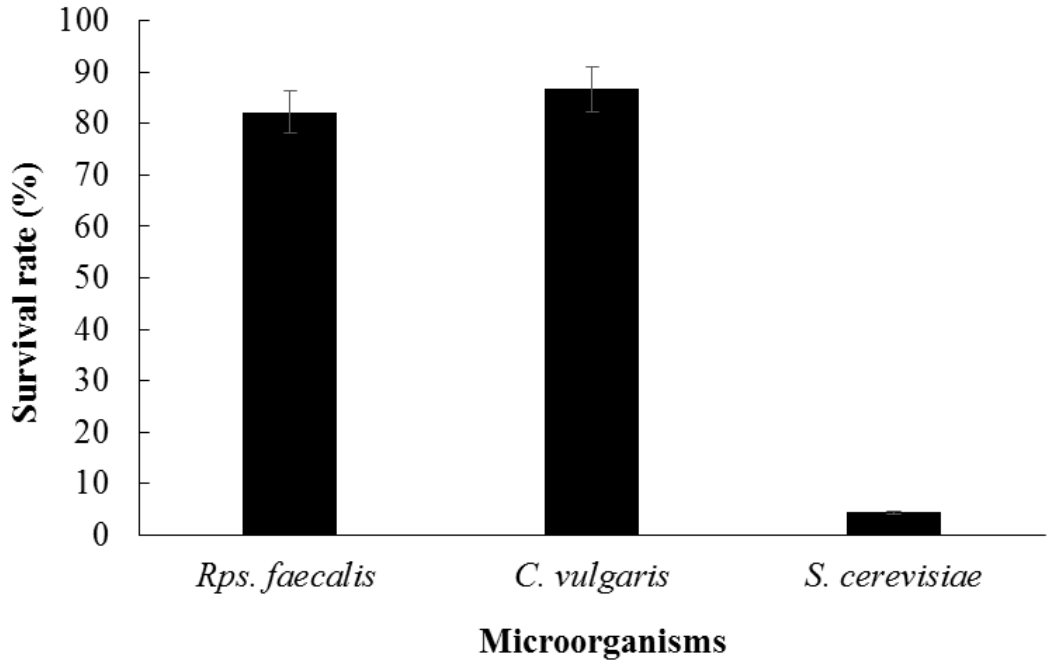

Fig. 6 Survival rate of the larvae *Streptocephalus sirindhornae* fed with *Rhodopseudomonas faecalis*, *Chlorella vulgaris* and *Saccharomyces cerevisiae*.

In all treatments, temperature fell within the range from 27.9 to 32.4 ºC. The pH of the rearing water were between 8.73 and 9.03 which are the weak basic pH. DO concentration of the yeast treatment had significantly lowest ($p < 0.05$) while those treated with *Rps. faecalis* and *C. vulgaris* were higher than 7 mg/L (Fig. 7A). The use of *S. cerevisiae* as feed showed the highest ammonia concentration of 1.54 ± 0.13 mg/L, followed by *Rps. faecalis* (0.66 ± 0.19 mg/L) and *C. vulgaris* (0.26 ± 0.03 mg/L) (Fig. 7B). As presented in Figs. 7C and 7D, the respective concentrations of nitrite and nitrate found in the water treated with *Rps. faecalis* were lower than the minimum threshold value at which the substances could be detected, whereas the highest concentrations were found in the algae treatment.

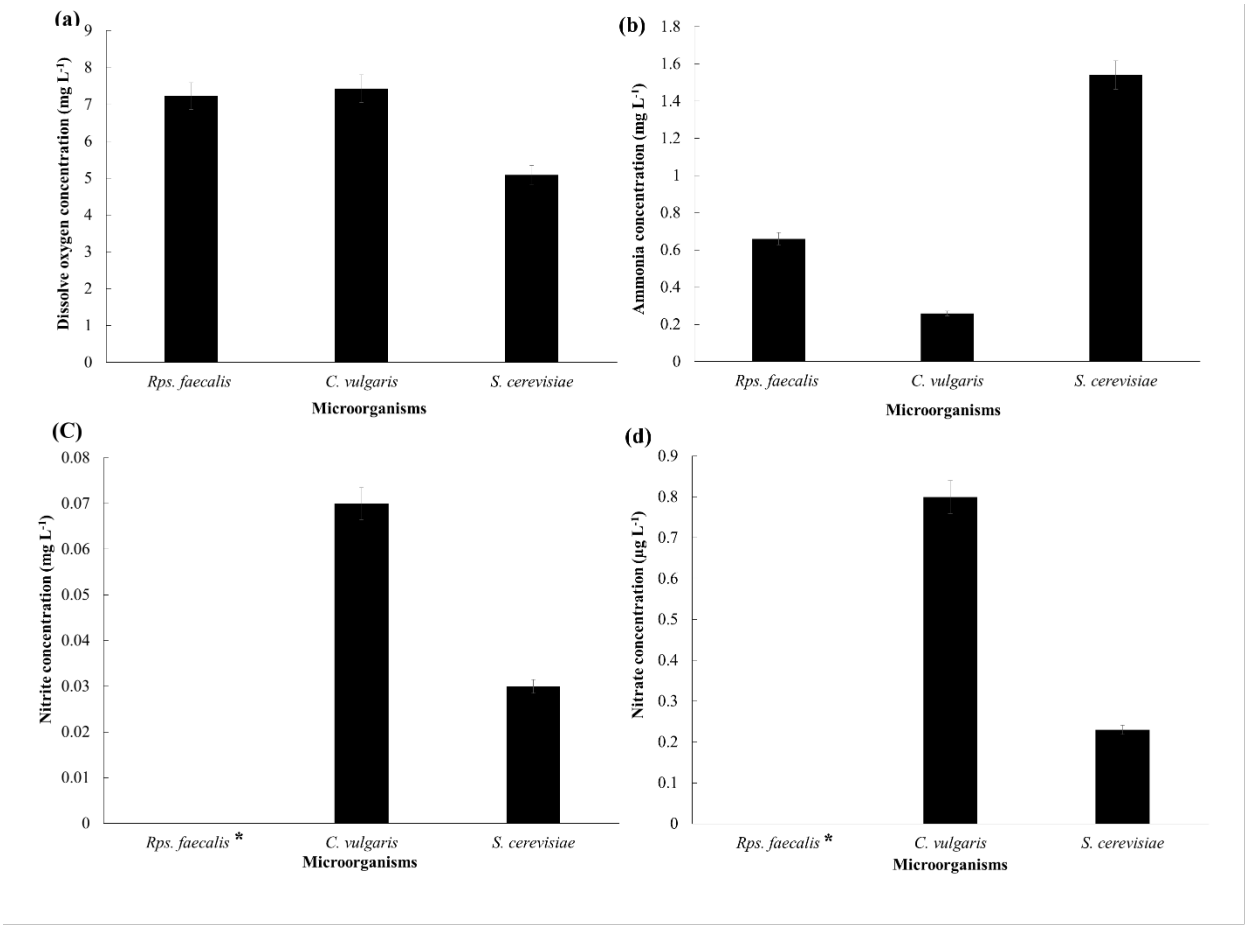

Fig. 7 Water quality of the rearing water of the larvae *Streptocephalus sirindhornae* fed with*Rhodopseudomonas faecalis*, *Chlorella vulgaris* and *Saccharomyces cerevisiae*. The parameters include dissolved oxygen concentration (a), ammonia concentration (b), nitrite concentration (c) and nitrate concentration (d). * denotes the respective concentration was lower than the minimum threshold value at which the substance could be detected.

7. Survival and growth performance of the sub-adults and adults

Sub-adult and adult fairy shrimp fed with *Rps. faecalis* had significantly higher survival rate (46.7%) than the other treatments (Fig. 8). Survival rate had almost two fold higher than the ones fed with *C. vulgaris* (26.7%). Survival rate of fairy shrimp fed with *S. cerevisiae* showed significant drop within 9 days of cultivation and it was the lowest ($p < 0.05$). At the end of experiment, survival rate of fairy shrimp fed with *S. cerevisiae* was 10%.

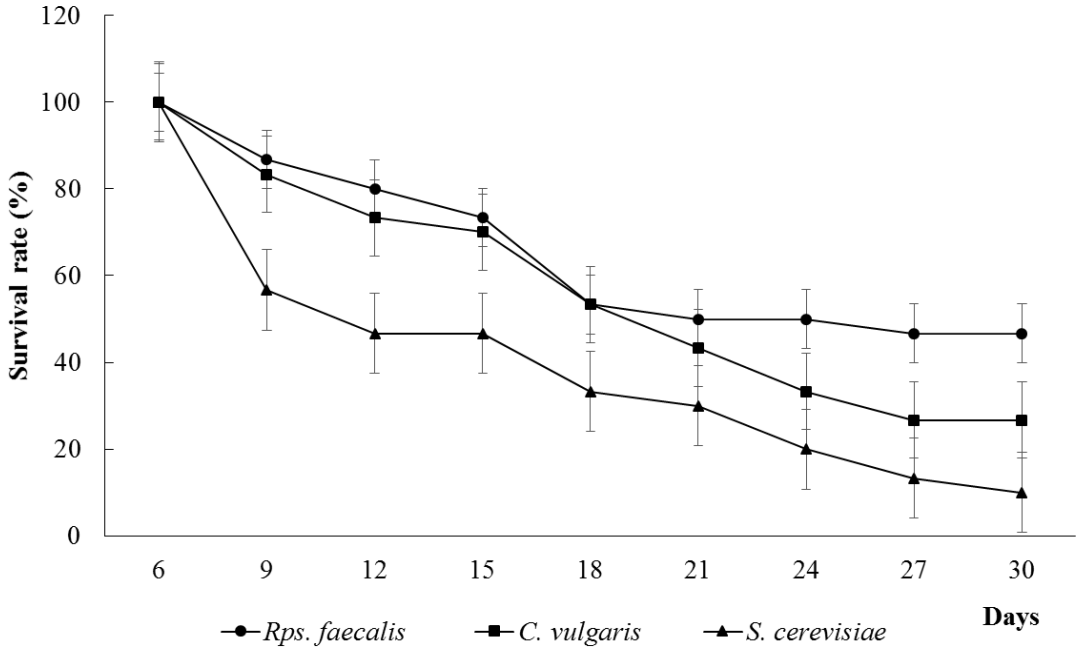

Fig. 8 Survival rate of sub-adult and adult fairy shrimp *Streptocephalus sirindhornae* fed with *Rhodopseudomonas faecalis*, *Chlorella vulgaris* and *Saccharomyces cerevisiae*.

As shown in Fig. 9, the highest growth rate of fairy shrimp fed with *Rps. faecalis* (0.99 ± 0.16 mm day^{-1}) was observed on day 12, whereas those fed with *C. vulgaris* had the highest growth rate (0.94 ± 0.18 mm day^{-1}) on day 15. The treatment with *S. cerevisiae* showed the lowest growth rate and the growth rate was quite stable during the experiment. Moreover, the duration of maturation of fairy shrimp fed with *Rps. faecalis* and *C. vulgaris* was less than that fed with *S. cerevisiae* (Table 7).

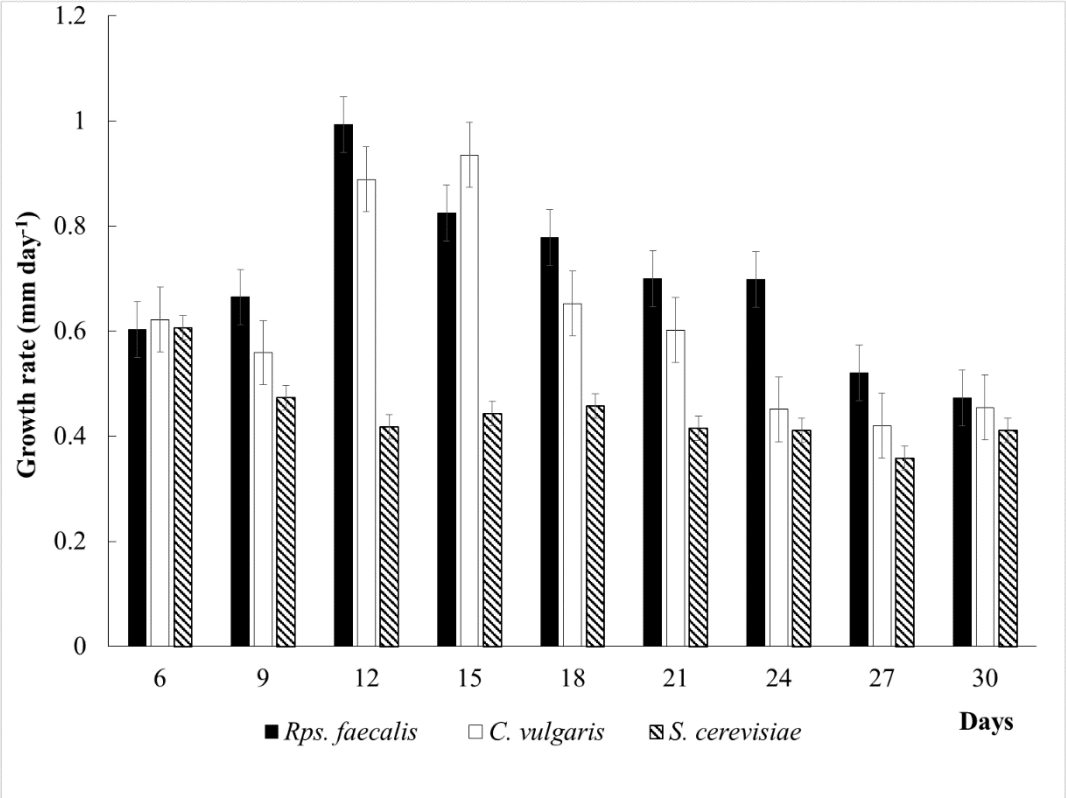

Fig. 9 Growth rate of sub-adult and adult fairy shrimp *Streptocephalus sirindhornae* fed with *Rhodopseudomonas faecalis*, *Chlorella vulgaris* and *Saccharomyces cerevisiae*.

Table 7 The duration of maturation in fairy shrimp fed with different microorganisms.

Microorganisms	Duration of maturation in fairy shrimp (day)
Rhodopseudomonas faecalis	10
Chlorella vulgaris	10
Saccharomyces cerevisiae	12

8. Water quality of the sub-adults and adults

The quality of rearing water was investigated 3 days interval before changing water except the treatment with *S. cerevisiae*. Water replacement in the yeast treatment was made frequently because of turbidity. As shown in Fig. 10A, DO concentration in the water treated with *S. cerevisiae* was between 1.4 and 4.4 mg/L, whereas it was higher than 5 mg/L in all the sampling periods when treated with *Rps. faecalis*. On the other hand, the rearing water of fairy shrimp fed with *S. cerevisiae* had significantly

higher ($p < 0.05$) ammonia concentration than those fed with *Rps. faecalis* and *C. vulgaris* (Fig. 10B). The highest concentrations of nitrite and nitrate were found in the water treated with *C. vulgaris* throughout the culture period (Figs. 10C and 10D). Nitrate and nitrite concentrations of the treatment with *C. vulgaris* were significantly different ($p < 0.05$) from those of the other treatments. During the experiment, temperature ranged from 24.8 to 26.8 ^{o}C, the pH were between 5.80 and 7.53.

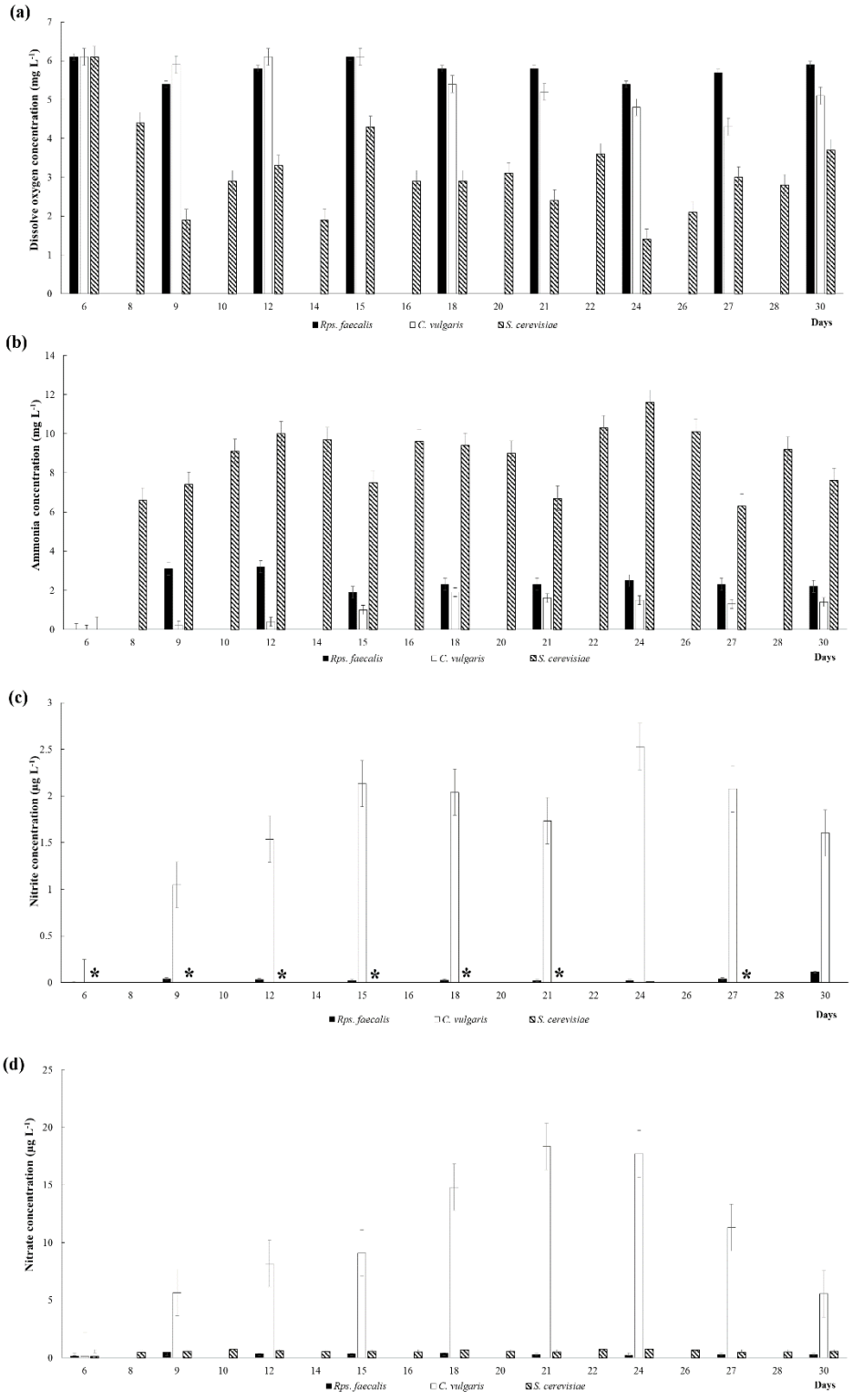

Fig. 10 Water quality of the rearing water of sub-adult and adult fairy shrimp *Streptocephalus sirindhornae* fed with *Rhodopseudomonas faecalis*, *Chlorella vulgaris* and *Saccharomyces cerevisiae*. The parameters include dissolved oxygen concentration (a), ammonia concentration (b), nitrite concentration (c) and nitrate concentration (d).

* denotes the respective concentration was lower than the minimum threshold value at which the substance could be detected.

Discussion

This study indicates the mass production of photosynthetic bacterium *Rps. faecalis* PA2 and carotenoid in the undiluted domestic wastewater without nutrient supplementation. The physical parameters including light intensity of 4,000 lux and agitation speed of 150 rpm were optimal for cultivation. The biomass obtained from the mass production showed the high carotenoid, protein and lipid contents with essential amino acids and essential fatty acids which meet the requirements for aquatic diets. Based on this study, *Rps. faecalis* PA2 grown in domestic wastewater is feasible to be used as a good feedstuff for aquaculture feed.

Survival and growth of the larvae, sub-adults and adults fairy shrimp

While much is known about the use of several algal species as diet in fairy shrimp. The present study has shown that photosynthetic bacterium *Rps. faecalis* could be used as an alternative diet in fairy shrimp cultivation. Survival rate of the larvae and adult fairy shrimp fed with *Rps. faecalis* were comparable with that of *C. vulgaris* which is considered to be the known diet used in larviculture and aquaculture. Moreover, the highest growth rate of fairy shrimp fed with *Rps. faecalis* was observed on day 12, whereas those fed with *C. vulgaris* had the highest growth rate on day 15 (Fig. 9), suggesting that this bacterium could enhance the growth of fairy shrimp. It has been reported that photosynthetic bacterial cell has a relatively high protein content consisting of all essential amino acids (Kornochalert et al., 2014). The methionine contents obtained from photosynthetic bacteria are comparable with that in the Food Agriculture Organization (FAO) reference (Kim & Lee 2000). Moreover, polyunsaturated fatty acids (PUFA) including omega-3 such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) and omega-6 were found in these bacteria (Loo et al., 2013). Compared to microalgae and yeast, photosynthetic bacteria contain ubiquinone-10 (CoQ10), carotenoids, vitamins and they have more digestible cell wall that resulted in facilitating nutrient and physiological growth factor assimilations (Tian et al., 2012). Importance of photosynthetic bacteria in aqua-hatcheries not only owes their nutritional attributes but they also have small size less than 3 μm meeting the feed size requirements ideally well for the larvae production (Saejung & Thammaratana, 2016). These may have been associated with the enhanced growth and survival obtained from the treatment with *Rps. faecalis*.

Water quality

The use of a high density, water re-use system is the alternative pond production system widely used in aquaculture. One of the major problems of this system is the removal of nitrogen waste produced from aquatic animals. Therefore, the present study evaluated the potential of each microbes in ammonia, nitrate and nitrite removal. Ammonia concentration found in the rearing water of larvae and adult fairy shrimp treated with *Rps. faecalis* and *C. vulgaris* was less than that found in the yeast experiment. It has been suggested that photosynthetic bacteria and unicellular green algae are capable of degrading ammonia nitrogen in the aquaculture water (Zhan & Liu 2013). Although the highest nitrate and nitrite concentrations were found in the water treated with *C. vulgaris*, survival of larvae was the highest. This could possibly be due to nitrate is considered to be relatively harmless to aquatic animals (Whitson et al., 1993). Additionally, larvae were exposed to the toxic nitrite in the short period (3 days), thus, survival rate was less affected. On the other hand, the high concentration of nitrite might have an adverse effect to the survival of sub-adult and adult fairy shrimp treated with *C. vulgaris* because of the long exposure time (30 days) to nitrite. Although water replacement was made 3 days interval, it was a partial replacement (only 50%), the remaining water still contained the amount of nitrite. As shown in Fig. 10D, the high nitrate concentration in the water treated with *C. vulgaris* might have been associated with the high nitrite concentration that resulted in decreasing survival rate of fairy shrimp compared with the treatment of *Rps. faecalis*.

This study indicates the use of *Rps. faecalis* in fairy shrimp culture did not only enhance survival and growth performance but it also improved rearing water quality *via* a reduction in ammonia, nitrate and nitrite. The results were related to many works which showed that phototrophic denitrifiers are able to decrease nitrate and nitrite concentrations in aquaculture system because they have the ability to utilize nitrate as an electron donor (Kim et al., 1999). Consequently, these bacteria are considered to be a potential component for recirculating aquaculture systems.

The disadvantages of baker's yeast in fairy shrimp culture

Our results clearly showed that the use of *S. cerevisiae* was not suitable for the fairy shrimp *S. sirindhornae*. Survival and growth performance of larvae and adult stages were not satisfactory. Maeda-Martinez et al. (1995) have suggested that the use of baker's yeast as basic food for rearing fairy shrimp *Thamnocephalus platyurus* and

Branchinecta lindahli showed the digestibility problem and nutritional deficiencies. Yeast fed to aquatic species showed significant reduction in growth, protein efficiency rate, nitrogen gain and they contain anti-nutritional factors which may hamper the performances of digestive tract. Mura et al. (1999) has found that fatty acid content of the fairy shrimp *Chirocephalus ruffoi* fed with baker's yeast was low compared with the other food. These phenomenon may explain the results of survival, growth performance and the delay of maturation stage of sub-adult and adult fairy shrimp fed with *S. cerevisiae*. Although the experiment was repeated, *S. cerevisiae* could not be used to raise the larvae. Therefore, *C. vulgaris* was used to feed the larvae for 5 days before feeding with each microbe in the subsequent studies. Although the larvae fed with *S. cerevisiae* showed the very low survival, the yeast treatment was not excluded in the sub-adult and adult experiments. This was because baker's yeast is occasionally fed to *S. sirindhornae* in some Thai agriculturists but the publication dealing with its effect on this fairy shrimp is scarce. Therefore, the results obtained in this study might be beneficial.

Besides the problem of growth performance, *S. cerevisiae* had the adverse effects on the water quality. Excessive turbidity of the rearing water occurred almost every day during the cultivation and the frequent water replacement could not overcome this incidence. Concurrent with turbidity of rearing water was the high ammonia concentration. Ammonia was released into the water by excretion from the fairy shrimp. This substance is highly toxic to aquatic species compared with nitrate and nitrite (Randall & Tsui, 2002). However, ammonia could be assimilated as a nitrogen source by microalgae and phototrophic bacteria as previously discussed but yeast could not that resulted in ammonia accumulation in the rearing water. These are the reasons why high ammonia concentration and low survival were found in the treatment with *S. cerevisiae*. In addition to the high ammonia concentration, the low level of DO was found in the water treated with *S. cerevisiae*. The DO concentration was lower than 5.0 mg/L in all the sampling periods. The level of ammonia is directly dependent on DO concentration and the low DO concentration can increase the toxic of ammonia. The significantly lower DO concentration in the treatment with *S. cerevisiae* could possibly be due to the required dissolved oxygen of yeast for respiration and growth, whereas *Rps. faecalis* grow anaerobically (Saejung & Apaiwong, 2015). Although *C. vulgaris* consumes oxygen for growth, it releases oxygen to environment *via* oxygenic photosynthesis (Peers, 2014). Moreover, osmotic stress might have been additionally

involved in the relatively lower survival rate of sub-adult and adult fairy shrimp fed with *S. cerevisiae* upon increased frequency of water changes. In our opinion, a flow-through system might have been more favorable for the feeding with yeast.

Suggestions for future research

The current study showed that the application of *Rps. faecalis* in fairy shrimp culture is technically feasible. Moreover, the mass production of this bacterium in the waste materials resulted in introducing the cost effective. The results of survival rate and growth enhancement of the fairy shrimp *S. sirindhornae* fed with *Rps. faecalis* were comparable to *C. vulgaris* which is the common diet used in fairy shrimp. Moreover, water quality of the rearing water treated with this bacterium was improved compared to *S. cerevisiae*. Therefore, further study of this bacterium in aquaculture is suggested. According to the results, the use of *S. cerevisiae* in fairy shrimp culture should be avoided. In the presence of yeast, survival and growth of fairy shrimp were not only decreased but excessive turbidity, high ammonia concentration and low DO content were also observed.

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Chapter 3

Output

1. **Saejung, C.** and Apaiwong, P. (2015) Enhancement of carotenoid production in the new carotenoid-producing photosynthetic bacterium *Rhodopseudomonas faecalis* PA2. **Biotechnology and Bioprocess Engineering** 20, 701-707. [ISI Impact Factor = 1.211]

2. **Saejung, C.** and Thammaratana, T. (2016) Biomass recovery during municipal wastewater treatment using photosynthetic bacteria and prospect of production of single cell protein for feedstuff. **Environmental Technology** 37, 3055-3061. [ISI Impact Factor 1.760]

Appendix

A1 **Saejung, C.** and Apaiwong, P. (2015) Enhancement of carotenoid production in the new carotenoid-producing photosynthetic bacterium *Rhodopseudomonas faecalis* PA2. **Biotechnology and Bioprocess Engineering** 20, 701-707. [ISI Impact Factor = 1.211]

A2 **Saejung, C.** and Thammaratana, T. (2016) Biomass recovery during municipal wastewater treatment using photosynthetic bacteria and prospect of production of single cell protein for feedstuff. **Environmental Technology** 37, 3055-3061. [ISI Impact Factor 1.760]

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RESEARCH PAPER

Enhancement of Carotenoid Production in the New Carotenoid-producing Photosynthetic Bacterium *Rhodopseudomonas faecalis* PA2

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Abstract Use of photosynthetic bacteria to produce carotenoids has increased considerably in recent decades; however, few studies have been conducted to identify additional carotenoid producers. In this study, *Rhodopseudomonas faecalis* PA2 was shown to be capable of carotenoid production, and factors influencing this production were identified. The maximum carotenoid content was observed at an initial pH of 7 in the presence of 0.8% malic acid, 0.4% yeast extract, and 0.05% Fe^{3+} under light at 4,000 lux. Fe^{3+} significantly enhanced carotenogenesis, resulting in a production rate of 2.5 mg/g/day and a reduction in time to maximum production from 12 to 8 days. The carotenoid content and carotenoid yield under modified conditions were 413 mg/L and 13 mg/g (mg total carotenoids per gram of dry cells), respectively, representing an increase of 117% relative to the original condition. The biomass and carotenoid productivity reached 4 g/L/day and 51.6 mg/L/day, respectively. To the best of our knowledge, this is the first study of carotenoid production by *Rps. faecalis* PA2. The results indicated that the productivity of this organism under the aforementioned conditions was comparable to that of previously described purple photosynthetic bacterial species.

Keywords: carotenoid, photosynthetic bacteria, *Rhodopseudomonas faecalis*, anoxygenic condition, carotenogenesis

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1. Introduction

Carotenoids are pigments located in the light-harvesting complexes of plants and microorganisms that provide photo-protection and light-absorbing functions [1,2]. There has been increased demand for carotenoids because of their potential applications, and their global market expanded by nearly $1.2 billion in 2010 [3]. Carotenoids can be used as antioxidants, precursors for vitamin A biosynthesis, anti-tumor and anti-cancer treatments, and to protect skin from the harmful effects of sunlight [4-6]. Recent research has shown that carotenoids are widely applicable in the pharmaceutical, nutraceutical and cosmetic industries [7]. In aquaculture, carotenoids are included in feed to enhance pigmentation, especially during salmon and ornamental fish cultivation, as well as to induce the immune system [8].

Currently, only a few commercial carotenoids can be produced *via* chemical synthesis, and these are prohibited in some cosmetic and food industries; accordingly, interest in carotenoids produced by microbes has recently increased considerably [9-13]. Some carotenoids have been found to be produced exclusively by photosynthetic bacteria, such as lycopene produced by *Rhodospirillum rubrum* [14]. Photosynthetic bacteria can synthesize carotenoids under anoxygenic conditions, which is cost effective. In addition, the cells of photosynthetic bacteria often contain several beneficial metabolites with high levels of total carotenoids, making them useful as aquatic animal feed [15]. Therefore, photosynthetic bacteria have the potential for use in carotenoid production in conjunction with biomass production.

Carotenoid production by photosynthetic bacteria is mainly limited to *R. rubrum*, *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* [14,16,17]. However, the newly isolated phototrophic purple non-sulfur bacterium

Rhodopseudomonas faecalis may be capable of carotenoid production [18]. Specifically, photosynthetic pigments of this species are known to include bacteriochlorophyll and carotenoids belonging to the spirilloxanthin series, but quantitative information is very scarce [18]. Despite the scant information regarding production of photosynthetic pigments by this species, it has recently been investigated for hydrogen production [19-21]. Hence, the present study was conducted to investigate carotenoid production by *Rps. faecalis* PA2 in the absence of oxygen. The effects of pH, light intensity, malic acid, yeast extract and metal ions on carotenogenesis were also evaluated.

2. Materials and Methods

2.1. Microorganism and media

Several photosynthetic bacteria were isolated from a wastewater treatment pond, and the highest growth and carotenoid-producing strain identified in preliminary studies, PA2, was used in this study. The 16S rDNA base sequence of PA2 was identical to that of *Rhodopseudomonas faecalis* strain gc^T with GenBank accession number AF123085 [18]. Glutamate-malate medium (GMM) was used for the pre-culture.

2.2. Optimization of the culture conditions for carotenoid production

Preliminary studies showed that GMM was suitable for the growth and carotenoid production of the strain when cultured at ambient temperature; thus, GMM was used as the basic medium. The experiment was carried out in 500-mL screw-capped production bottles filled completely with GMM to generate anoxygenic conditions. The parameters for the seed culture were as follows: inoculant age of 48 h, OD_{660} of 0.5, inoculant volume of 10% (v/v). The culture was incubated under static light conditions at ambient temperature (approximately 30°C) for 12 days. At each 24-h interval, samples were collected for total carotenoid analysis and to determine the growth based on the dry cell weight.

To explore the effects of initial pH on carotenoid production, culture medium adjusted to pH 6.0 ~ 9.0 was used for cultivation at a light intensity of 2,000 lux. To determine the optimum light intensity, the strain was cultivated at 1,000 ~ 4,000 lux (Lux Meter HI 97500; Hanna Instruments, Inc., Woonsocket, RI, USA). The effects of malic acid were investigated by adding 0.2 ~ 3.2% (w/v) to the basic medium prior to inoculation. Medium without malic acid was used as a control. The effects of initial yeast extract concentrations of 0.4 ~ 2.0% (w/v) were also investigated based on comparison to basic medium without yeast extract as a control. To determine the effects of metal ions (Zn^{2+}, Cu^{2+}, Fe^{2+} and Fe^{3+}), 0.05% (w/v) of each metal was added to the medium. The optimum factor from each experiment was used in each subsequent experiment until all factors were optimized.

2.3. Analytical methods

Growth of the strain was determined based on the dry cell weight [22]. Carotenoid extraction was conducted by centrifuging the culture broth at 6,000 rpm and 4°C for 20 min (Himac CR20B2; Hitachi, Tokyo, Japan). The pellets were then washed twice with 0.9% NaCl and extracted using methanol-acetone (2:3 v/v) solvent. Cell extraction was repeated until the sample was colorless, after which the absorbance of the extract at 480 and 770 nm was measured using a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Total carotenoid content was calculated by Equation (1) [23], while bacterial biomass and carotenoid content were calculated statistically. All data were expressed as the means ± standard deviation (SD).

$$\text{Total carotenoid content} = (A_{480} - 0.1\ A_{770}) \times 0.385 \quad (1)$$

where A_{480} and A_{770} were the absorbance of the extract at 480 and 770 nm, respectively.

The results are presented as the dry biomass at any time and at the end of growth (g/L), total carotenoid content at any time and at the end of growth (mg/L total carotenoids of culture broth), specific growth rate (/day), carotenoid production rate (mg/g/day), carotenoid yield determined as the amount of carotenoids produced per unit of dry weight of cells (mg total carotenoids per gram of dry cells), biomass productivity (g/L/day) and carotenoid productivity (mg/L/day).

3. Results and Discussion

Fig. 1 shows the time-course of growth and carotenoid production of *Rps. faecalis* PA2 under baseline conditions (GMM, pH 6.8, light intensity = 2,000 lux). The highest carotenoid content of 190 mg/L was produced during the stationary phase of growth because carotenoids are secondary metabolites [24,25]. Additionally, the carotenoid content was significantly higher at 180 h, and the results suggested that this increase would continue with additional incubation time. Therefore, the strain was cultivated for longer than 180 h in subsequent experiments.

3.1. Effect of initial pH on growth and carotenoid production

As shown in Fig. 2A, the initial pH significantly influenced

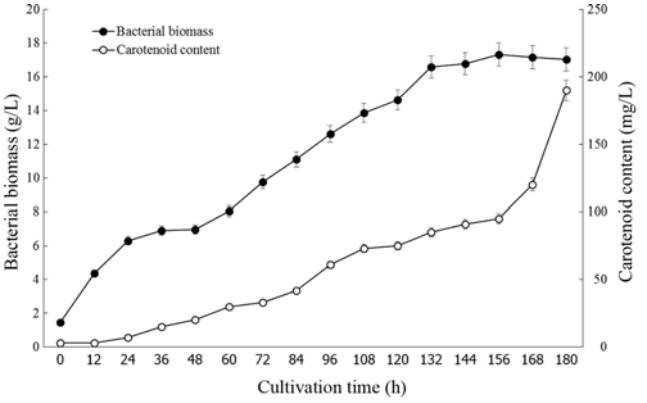

Fig. 1. Time-course study of growth and carotenoid production of *Rps. faecalis* PA2. Error bars represent standard deviations of the means.

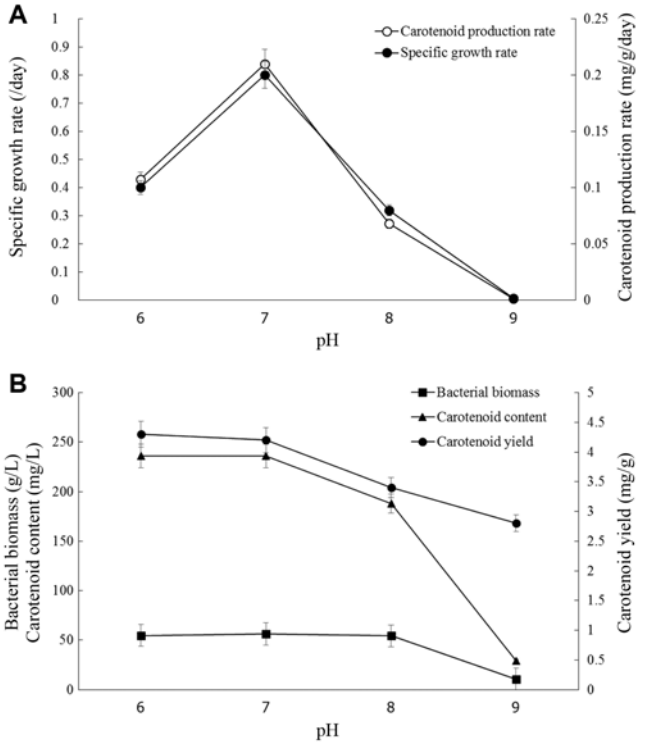

Fig. 2. Effect of initial pH on growth and carotenoid production of *Rps. faecalis* PA2. (A) Specific growth and carotenoid production rates; (B) bacterial biomass, carotenoid content, and carotenoid yield. Error bars represent standard deviations of the means.

the growth and total carotenoid production of *Rps. faecalis* PA2. Specific growth and carotenoid production rates increased when the pH increased to 7, while they decreased at higher pH. As shown in Fig. 2B, the highest carotenoid content of 236 mg/L was observed at pH 6 and 7, while the highest biomass and carotenoid production rates were observed at pH 7. In contrast, the lowest specific growth and carotenoid production rates were observed at pH 9. Strong acidic and alkaline pH causes the denaturation of

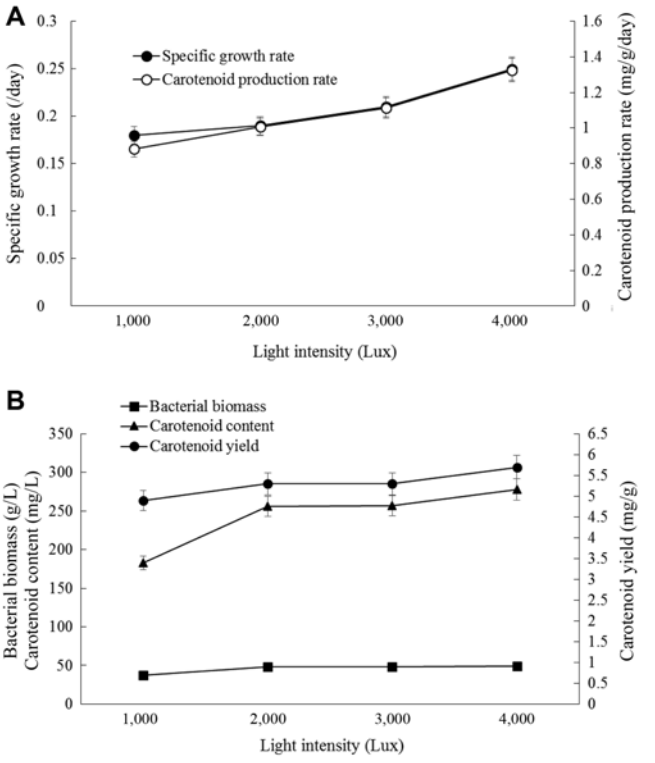

Fig. 3. Effect of light intensity on growth and carotenoid production of *Rps. faecalis* PA2. (A) Specific growth and carotenoid production rates; (B) bacterial biomass, carotenoid content, and carotenoid yield. Error bars represent standard deviations of the means.

enzymes, distortion of cell structure and suppression of carotenoid synthesis, resulting in slow growth and low carotenoid content [26].

3.2. Effect of light intensity on growth and carotenoid production

As shown in Figs. 3A and 3B, exposure to a light intensity of 4,000 lux resulted in the highest specific growth rate of 0.25/day, while bacterial biomass was not significantly higher than at 2,000 and 3,000 lux. These findings suggest that the highest light intensity (4,000 lux) stimulated energy absorption and accelerated the phototrophic bacterial growth relative to the lower light intensity. When there is excess light energy, bacteria cannot use all of the light energy for cell growth because of photo-inhibition, resulting in termination of biomass accumulation [27]. In contrast, higher light intensity was associated with higher carotenoid biosynthesis. Specifically, light at 4,000 lux produced the highest carotenoid content and carotenoid production rates of 278 mg/L and 1.3 mg/g/day, respectively. This is not surprising since application of high light levels is known to be one of the best strategies for maximization of carotenoid accumulation in cells. Specifically, carotenoid accumulation is associated with phytoene synthase and carotenoid

hydroxylase, which are activated in the presence of increased light intensity [28]. A similar phenomenon has been reported in microalgae [29]. The exposure of *Rps. faecalis* PA2 to light at 1,000 lux resulted in the lowest carotenoid yield, which is in accordance with the results of a previous study showing that carotenoid biosynthesis was repressed in the dark and under low light intensity [30].

3.3. Effect of malic acid concentration on growth and carotenoid production

The concentration of malic acid is another important factor because it is sequentially produced in the metabolic pathway and associated with carotenoid formation. As shown in Fig. 4A, as the concentration of malic acid increased, specific growth and carotenoid production increased, with the maximum levels of 44 g/L biomass and 285 mg carotenoid/L, respectively, occurring at 0.8% (w/v), above which production decreased (Fig. 4B). The results of the present study showed that the addition of malic acid (0.2 ~ 0.8%) supported higher biomass and carotenoid content relative to the control. This was likely because malic acid is an intermediate of the tricarboxylic acid (TCA) cycle, which is essential to metabolism and carbon skeleton formation during carotenoid and lipid biosynthesis [31]. The TCA cycle is also involved in the production of free radicals and singlet oxygen, which enhance carotenogenesis [32]. Previous findings have also reported that malic acid was a major component involved in stimulating carotenoid synthesis in the photosynthetic bacteria, *Rb. sphaeroides* and *Rubrivivax gelatinosus* [22,33]. However, specific growth and carotenoid production rates decreased when the malic acid levels reached 1.6 and 3.2%, respectively, indicating that levels above this threshold might exert a negative effect on the growth of bacteria.

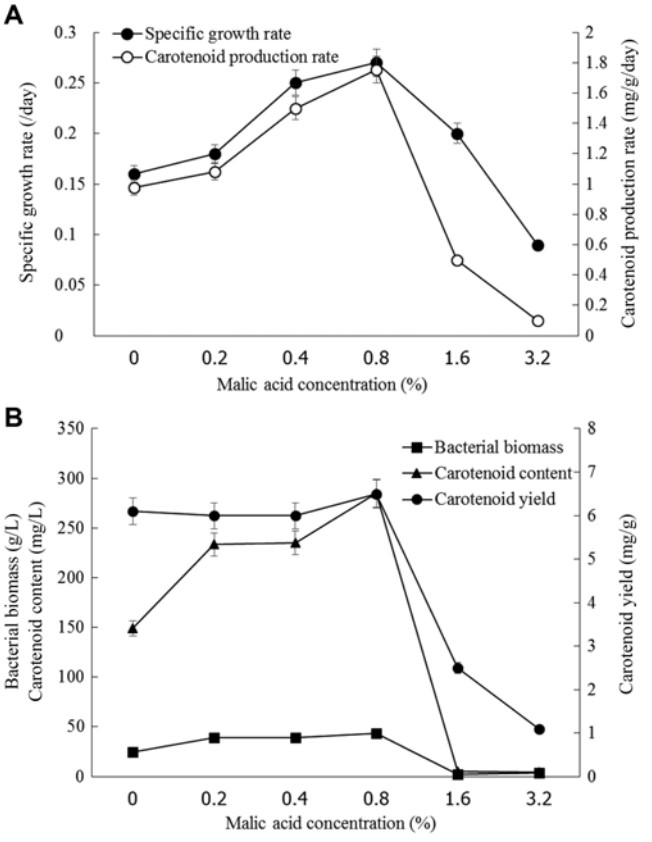

Fig. 4. Effect of malic acid concentration on growth and carotenoid production of *Rps. faecalis* PA2. (A) Specific growth and carotenoid production rates; (B) bacterial biomass, carotenoid content, and carotenoid yield. Error bars represent standard deviations of the means.

3.4. Effect of yeast extract concentration on growth and carotenoid production

The maximum specific growth and carotenoid production rates increased with increasing yeast extract concentration up to 0.4% (w/v), above which they sharply decreased (Fig. 5A). As indicated in Fig. 5B, the highest carotenoid content was observed when 0.4% yeast extract was added to the medium. Growth and carotenogenesis were significantly suppressed when the initial yeast extract concentration was 2.0%. These findings indicated that carotenoid bio-

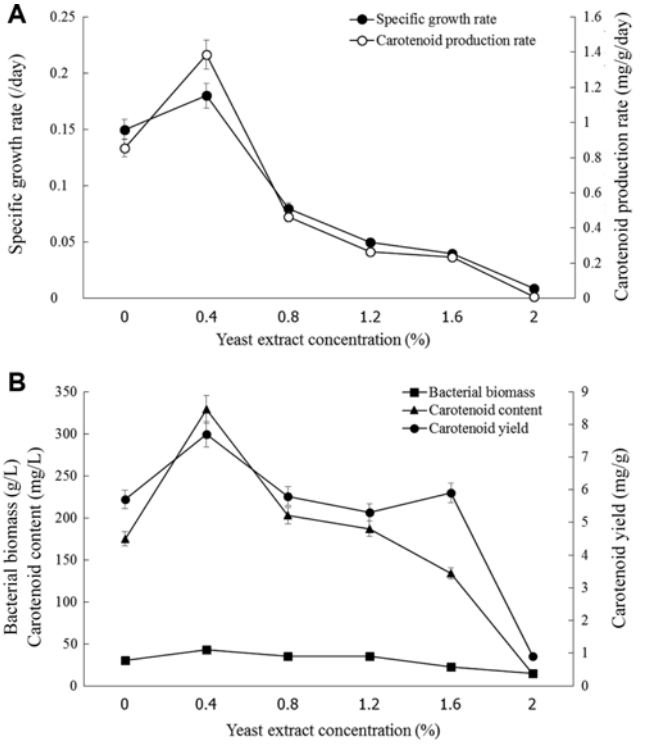

Fig. 5. Effect of yeast extract concentration on growth and carotenoid production of *Rps. faecalis* PA2. (A) Specific growth and carotenoid production rates; (B) bacterial biomass, carotenoid content, and carotenoid yield. Error bars represent standard deviations of the means.

synthesis could be induced by yeast extract supplementation at low levels [34]. Additionally, bacterial growth and carotenoid content were observed in medium without yeast extract. It is believed that the bacteria used not only yeast extract, but also other compounds in the medium as vitamin and nitrogen sources to support growth.

3.5. Effect of metal ions on growth and carotenoid production

Metal ions were found to induce and suppress carotenogenesis. Fe^{3+} showed the highest positive effect on growth (0.2/day) and carotenoid production (2.5 mg/g/day) rates, resulting in carotenoid contents of 413 mg/L (Fig. 6A) and a yield of 13 mg/g (Fig. 6B). When compared with the other metal ions, Fe^{3+} and Fe^{2+} led to significant increases in carotenoid biosynthesis. It is well known that ferrous ions are cofactors for carotenogenic enzymes that stimulate the carotenoid biosynthetic pathway [35]. Similarly, ferrous ion is assumed to be involved in uptake systems and intracellular binding sites, resulting in increased carotenoid accumulation [36]. It is also possible that the generation of hydroxyl radical *via* the Fenton reaction of ferrous ion ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^- + HO^*$) induces carotenogenesis [37]. Many studies have reported that copper ions improved growth and carotenoid biosynthesis in microorganisms [38,39]; however, the opposite was observed in the present study. The inhibitory effect of copper ion observed in the present study was likely due to the different concentration used here relative to previous studies.

3.6. Microbial growth and carotenoid production kinetics

The optimum factor from each experiment was used in subsequent experiments until all factors were examined. Figs. 7A and 7B compare the growth and carotenoid production curves of *Rps. faecalis* PA2 observed during the different experiments. The growth of bacteria began after an initial lag of 18 ~ 20 h, then increased slowly during the first 2 days. Bacterial biomass increased exponentially with time for 9 ~ 10 days, then stabilized (Fig. 7A). As indicated in Fig. 7B, a large amount of carotenoids were produced at the end of the exponential to stationary growth phase because the pigments were secondary metabolites. However, carotenoids are pigments that accumulate in photosynthetic bacterial cells to provide light absorption and photoprotection functions; therefore, they are present at elevated levels during exponential growth as well.

Biomass was highest in the pH experiment, but then decreased in subsequent experiments (Fig. 7A). Conversely, the lowest carotenoid was observed in the pH experiment, while higher values were observed in subsequent experi-

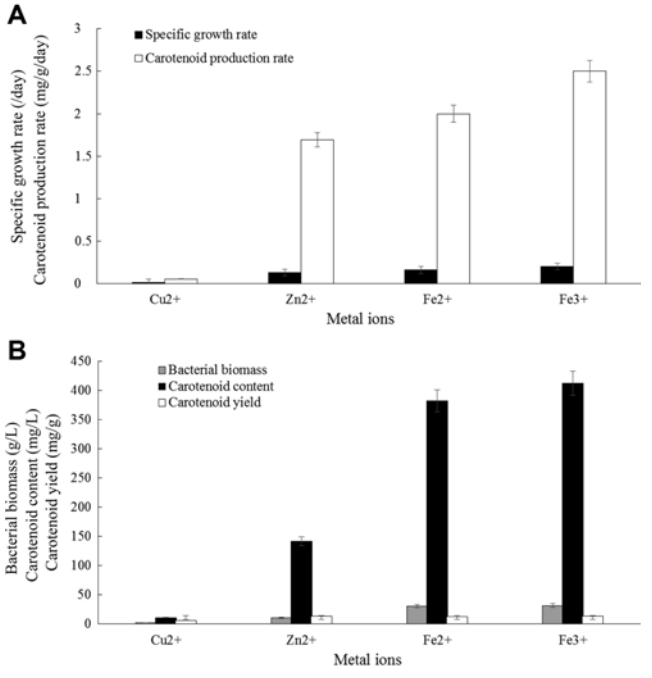

Fig. 6. Effect of 0.05% metal ions on growth and carotenoid production of *Rps. faecalis* PA2. (A) Specific growth and carotenoid production rates; (B) bacterial biomass, carotenoid content, and carotenoid yield. Error bars represent standard deviations of the means.

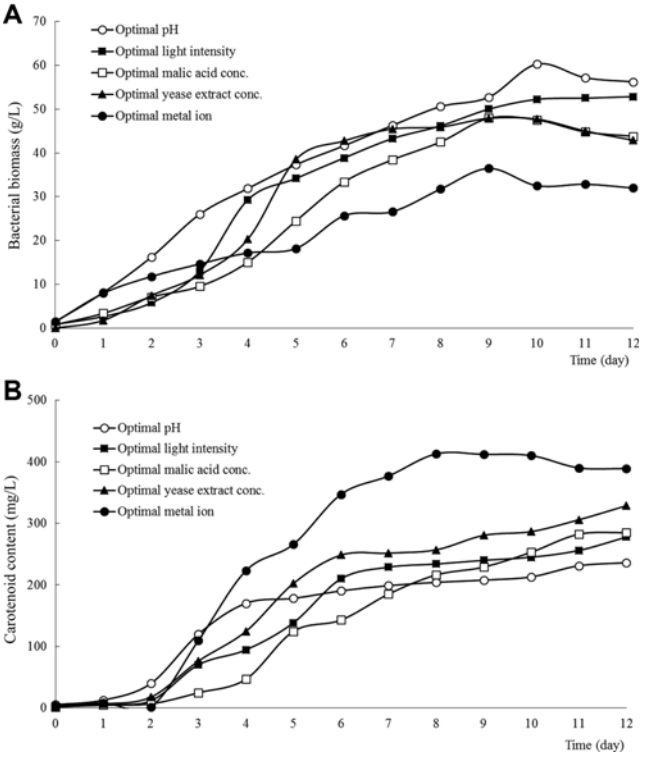

Fig. 7. Bacterial growth (A) and carotenoid production (B) trends obtained under optimal conditions for each factor (initial pH of 7; light intensity of 4,000 lux; 0.8% malic acid; 0.4% yeast extract; and Fe^{3+} supplement).

Table 1. Bacterial biomass and total carotenoid production from purple photosynthetic bacterial species under various conditions

Photosynthetic bacterial species	Carotenoid content		Biomass (g/L)	Carotenoid productivity (mg/L/day)* (mg/g/day)**	Biomass productivity (g/L/day)	Reference
	(mg/L)	(mg/g)				
Rhodopseudomonas faecalis PA2	413	13	32	51.6* / 1.6**	4	Present study
Rhodopseudomonas sp.	1.46	NA	ND	0.73*	ND	[40]
Rhodopseudomonas sp.	ND	4.08	3.26	1.36*	1.09	[42]
Rps. palustris	ND	1.10	2.5	0.27**	0.62	[43]
Marichromatium sp.	ND	3.28	3.83	1.64*	1.91	[42]
Rhodobacter sphaeroides	17.25	ND	ND	3.45*	ND	[16]
Rhobrivivax gelatinosus	ND	1.2	5.6	0.24**	1.12	[44]

ND: no data.

ments (Fig. 7B). These findings were likely because the pH experiment was conducted at 2,000 lux, while the other experiments were performed at 4,000 lux. These findings suggested that strong light intensity was not favorable for the growth of *Rps. faecalis* PA2, but that it stimulated synthesis of carotenoids for photochemical protection under excessive light conditions [40,41].

We also investigated the cultivation time at which the highest carotenoid content was obtained under the optimum conditions for each factor. Upon Fe^{3+} supplementation, the highest carotenoid content was observed within 8 days, while the highest levels were observed at the end of the cultivation period in the other experiments (day 12). Based on these results, the addition of Fe^{3+} reduced the cultivation time required for carotenoid production and increased the carotenoid production rate.

Table 1 summarizes the total carotenoid production obtained from purple photosynthetic bacteria under various conditions. The cultivation time for the maximum carotenoid production presented in previous studies was between 3 and 7 days, while this occurred on day 8 in the present study; thus, the results were calculated in terms of productivity (carotenoid produced/cultivation time) to allow comparison among studies. The total carotenoid productivity (51.6 mg/L/day and 1.6 mg/g/day) and biomass productivity (4 g/L/day) of this strain were comparable to those that have been reported for other purple photosynthetic bacterial species. Future studies should be conducted to determine the carotenoid composition of this strain.

4. Conclusion

This study revealed the potential use of the purple photosynthetic bacteria *Rps. faecalis* PA2 for carotenoid production under anoxygenic conditions. The results showed that an initial pH of 7, light intensity of 4,000 lux, 0.8% malic acid, 0.4% yeast extract and 0.05% Fe^{3+} were optimal for enhancing carotenogenesis in this strain. The addition of Fe^{3+} not only increased carotenoid content, but also reduced cultivation time, resulting in a carotenoid content of 413 mg/L and a carotenoid yield of 13 mg/g. The results of this study indicate that it is feasible to use *Rps. faecalis* PA2 for practical application.

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Biomass recovery during municipal wastewater treatment using photosynthetic bacteria and prospect of production of single cell protein for feedstuff

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ABSTRACT

Utilization of photosynthetic bacteria (PSB) for wastewater treatment and production of biomass for economical single cell protein production is a feasible option. In this study, *Rhodopseudomonas* sp. CSK01 was used for municipal wastewater treatment and the effect of initial pH, light intensity and additional carbon source was investigated. Optimum chemical oxygen demand (COD) removal and biomass production were achieved when the initial pH and light intensity were 7 and 4000 lux, respectively. The specific growth rate, biomass yield and biomass productivity were found to be 0.4/d, 3.2 g/g COD and 2.1 g/L/d, respectively, which were improved by 100%, 167% and 200% relative to the original condition. Under the optimal conditions, COD removal reached 85% and maximum biomass was 6.2 g/L accomplished within three days of cultivation. The biomass had a relatively high protein content (60.1%) consisting of all essential amino acids. The contents of histidine, lysine, phenylalanine and leucine were superior to those of the previously described PSB. Results showed that COD removal was not improved in the presence of additional carbon sources (glucose, sucrose and malic acid). The addition of malic acid significantly increased the biomass accumulation by 279% relative to the original condition, whereas COD removal was declined due to carbon catabolite repression. In this study, PSB biomass recovery and catabolite repression are proposed in municipal wastewater treatment by *Rhodopseudomonas* sp.

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Introduction

Interest in biological wastewater treatment has increased considerably since it possesses the economic advantage of both capital investment and operating costs. Photosynthetic bacteria (PSB) are one group of microorganisms utilized for the wastewater treatment system.[1–5] Traditional biological wastewater treatment generates large amounts of residual sludge to degrade organic carbonaceous matter to CO_2, whereas PSB can assimilate organic pollutants into cellular constituents. Moreover, PSB can assimilate CO_2 photoautotrophically, suggesting that they are the candidate for greenhouse gas reduction. However, there has been a small amount of reports on municipal wastewater treatment by PSB compared to industrial wastewater treatment.[6,7]

To improve waste removal, the manipulation of chemical and physical factors needed for the growth of PSB is required. Light is an important physical factor for the growth of PSB since light is used as an energy source *via* photosynthesis. Many work have shown that the growth of PSB was repressed under dark condition.[8,9] Additionally, bacterial photosynthesis is regulated by light intensity. Previous research[10] has shown that higher light intensity can increase the biomass production of PSB. However, it has been reported that light intensity above the threshold might exert a negative effect on the biomass accumulation of bacteria.[11] Furthermore, the supplement of carbon sources in biological wastewater treatment seems to improve the performance of biological wastewater treatment due to promoting the growth of degrading microorganisms. This strategy has been widely used to eliminate inorganic phosphorus and nitrogen from wastewater. It has been reported that the addition of external carbon sources enhanced denitrification and phosphorus removal in wastewater.[12,13] However, additional carbon source required for wastewater treatment by PSB is not well examined. Therefore, the investigation of optimum light intensity and additional carbon sources seems to be an interesting aspect and they vary depending upon the species of PSB.

Microbial protein, known as single cell protein (SCP), is widely accepted for human consumption and animal feed since there has been increased demand for protein supplement because of the shortage of protein originating from plants and animals. In addition to

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algae and yeast, PSB is utilized for SCP production because they contain significantly large amounts of carotenoid pigments, biological cofactors, vitamins, ubiquinone-10 (CoQ10), protein, amino acids and fatty acids. [14–16] Compared with algae and yeast, PSB have more digestible cell wall. Moreover, they can grow easily on a wide range of waste materials, which is the important characteristic of microbes used for SCP production in order to minimize production cost.

Hence, the objectives of this study are to investigate municipal wastewater treatment by PSB in association with biomass production and determine the protein content and amino acid composition of the biomass produced. The effects of pH, light intensity and additional carbon source are also discussed.

Materials and methods

Materials

Rhodopseudomonas sp. CSK01 isolated from facultative pond was used in this study.

The strain was cultivated in glutamate-malate medium under anoxygenic condition at ambient temperature (26–30°C) and light intensity at 2000 lux. Undiluted municipal wastewater was collected from the facultative pond of the local wastewater treatment system located in Khon Kaen province, Thailand. The wastewater was filtered through the nylon filter with a size of 60 µm in order to separate the large particles such as zooplankton and phytoplankton. Prior to use, it was sterilized at 121°C for 30 min. To analyze the water quality, closed reflux method (standard method part 5220°C), Kjedahl method and stannous chloride method were used to evaluate the chemical oxygen demand (COD), total kjeldahl nitrogen (TKN) and total phosphorus (TP), respectively. Total solids (TS) was analyzed by drying at 103–105°C and total suspended solids (TSS) was analyzed according to standard method part 2540 D. Dissolved oxygen (DO) and electrical conductivity (EC) were measured using HI9829 Multiparameter (Hanna Instruments, Woonsocket, Rhode Island, USA). [17] The COD, TKN, TP, TS, TSS, DO and EC were 5000, 176, 127, 346, 15, 1.4 mg/L and 723 µS/m^2, respectively. The initial pH was 6.8.

Experimental setup

The pH (5, 6, 7, 8 and 9), light intensity (1000, 2000, 3000 and 4000 lux) and 1% additional carbon sources (malic acid, glucose and sucrose) were optimized. Experiments were carried out in a batch reactor. Prior to the experiment, the reactor was sterilized at 121°C for 30 min. PSB was inoculated into the wastewater and the parameters of the inoculum were as follows: inoculant age of 48 h, OD_{660} of 0.5 and inoculant volume of 10% (v/v). The culture was illuminated and incubated under anoxygenic condition at ambient temperature. The treatment time of the batch reactor was 10 days. The strain cultivated in the wastewater under pH 6.8 and light at 2000 lux was defined as the original condition. Replicate experiments were conducted to obtain accurate data.

Analytical methods

The culture was centrifuged at 6000 rpm at 4°C for 30 min (Himac CR20B2; HITACHI, Tokyo, Japan). The supernatant was used to analyze COD by using the closed reflux method, and the collected bacterial cells were prepared for lyophilization using a freeze-dryer (Freezone 2.5L; LABCONCO, Kansas City, United States).[18] Biomass yield was determined as the amount of biomass produced/unit of COD consumed. Biomass productivity was defined as the maximum biomass/the cultivation time at which the maximum biomass was produced. The lyophilized biomass was used to evaluate the crude protein by the Kjeldahl method following the official AOAC 991.20 method,[19] and amino acid composition was determined by using an in-house method based on the AOAC Official Method 994.12, 988.15 (2000) and detected by GC-MS (GC Model 6890/MS Model 5973; Agilent Technologies, CA, United States) using a Zebron ZB-AAA (10 m × 0.25 mm id, 0.25 µm film thickness) column (Phenomenex).[20] The flow rate was 8 mL/h. Column temperature was held at 59°C for 0–50 min, then increased to 65°C for 50–90 min. Pump regenerant was 0.2 M NaOH for 0–5.35 min. Elution buffers were 0.2 M sodium citrate elution buffer 1 (pH 4.25, 5.35–50 min) and 0.14 M sodium citrate elution buffer 2 (pH 5.3, 50–90 min) with 2-propanol.

Scanning electron microscopic examination

The fresh culture of *Rhodopseudomonas* sp. CSK01 was centrifuged and washed twice with 0.1 M phosphate buffer saline (PBS) of pH 7.4. The bacterial suspension was spread over the surface of a 13-mm diameter 0.2 µm polycarbonate nucleopore membrane filter. The suspension was allowed to air dry, then fixed in 2.5% glutaraldehyde in 0.1 M PBS pH 7.4 at 4°C overnight, followed by washing with 0.1 M PBS pH 7.4 at 4°C for 5 min. Graded ethanol (30%, 50%, 70%, 90% and 100%) series were used for dehydrating the bacterial cell held on the filter. The dehydration in 90% and 100% ethanol was carried out two and three times, respectively. The specimen was passed through each ethanol

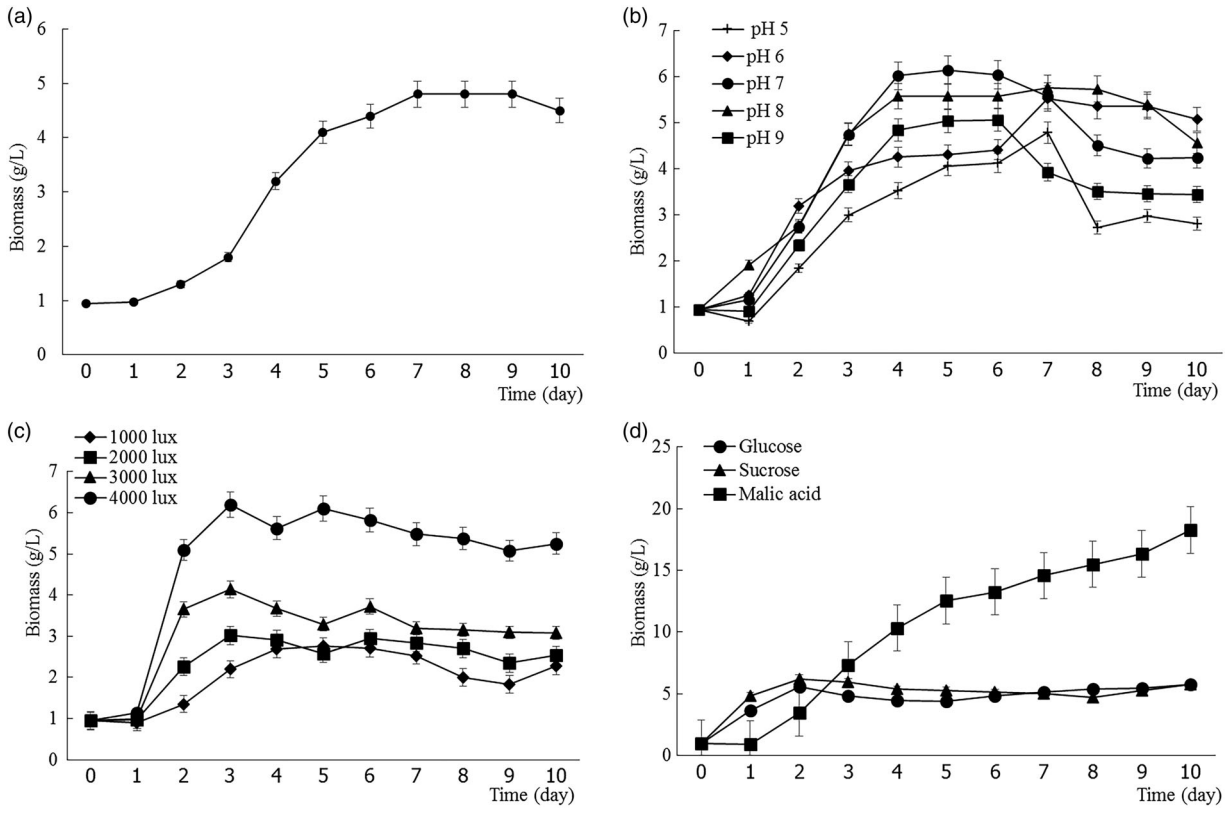

Figure 1. Growth profile of Rhodopseudomonas sp. CSK01 grown in the undiluted municipal wastewater under pH 6.8 and light at 2000 lux (defined as original condition) (a), light at 2000 lux and various initial pH (b), pH 7 and various light intensity (c) and pH 7, light at 4000 lux and various additional carbon sources (d).

concentration at 30-min intervals. It was allowed to air dry, then mounted onto aluminum stubs using colloidal carbon and sputter-coated with a gold layer. The specimen was examined under Scanning Electron Microscope LEO 1450VP (LEO Electron Microscopy Ltd, Cambridge, United Kingdom).

Results and discussion

The strain grown in wastewater in the original condition (pH 6.8 and light at 2000 lux) is shown in Figure 1(a), bacterial growth increased gradually with time up to seven days, and then stabilized. COD removal, COD consumed and biomass yield are given in Table 1(a).

Effect of initial pH on biomass production and COD removal

In practical wastewater treatment, it is difficult to control the pH range. Besides, the preliminary study showed that this strain could grow well in a wide pH range. Thus, this study did not control the pH range during the treatment and it would be more economical and desirable to employ in the real system. The effect of initial pH was selected to investigate the growth of bacteria in wastewater. As shown in Figure 1(b), growth of the strain in neutral and weak basic pH (7–9) was higher than that in acidic pH (5–6). Padan et al. [21] have reported that

Table 1. Specific growth rate (μ), biomass production (X), COD consumed (S), biomass yield ($Y_{x/s}$), biomass productivity (Q_p) and COD removal obtained from *Rhodopseudomonas* sp. CSK01 under original and optimized conditions.

Optimized factors	μ (/day)	X (g/L)	S (g/L)	$Y_{x/s}$ (g/g COD)	Q_p (g/L/day)	COD removal (%)
(a) Original condition						
None	0.2	4.8	4	1.2	0.7[a]	85
(b) Effect of initial pH						
pH 5	0.3	4.8	1.6	3.0	0.7[a]	78
pH 6	0.2	5.7	1.6	3.7	0.8[a]	80
pH 7	0.4	6.1	5.5	1.1	1.2[b]	94
pH 8	0.3	5.8	4.7	1.2	0.8[a]	90
pH 9	0.3	5	4.7	1.1	0.8[c]	88
(c) Effect of light intensity						
1000 lux	0.1	2.8	1.9	1.4	0.6[b]	86
2000 lux	0.3	3	1.8	1.7	1[d]	85
3000 lux	0.3	4.1	1.6	2.6	1.4[d]	81
4000 lux	0.4	6.2	1.9	3.2	2.1[d]	85
(d) Effect of additional carbon source						
Malic acid	0.6	18.2	1.4	13.5	1.8[e]	23
Glucose	0.8	5.5	3.3	1.6	2.8[f]	71
Sucrose	0.8	6.2	3.2	1.9	3.1[f]	71

[a]Calculated from seven days; [b]calculated from five days; [c]calculated from six days; [d]calculated from three days; [e]calculated from 10 days; [f]calculated from two days.

bacteria have limitation to their acidity tolerance. In the presence of low external pH, the concentration of H^+ is greater outside than inside, resulting in the movement of H^+ into the cytoplasm which lowers internal pH. The low cytoplasmic pH can harm bacteria by disturbing the plasma membrane or disrupting the activity of enzymes and membrane transport proteins. Furthermore, previous investigations [22,23] have shown that changes in the pH of the medium are likely to alter the ionization of nutrient molecules, thus reducing their availability to the microorganisms. The highest biomass production, specific growth rate and biomass productivity were achieved at pH 7. A similar study found that PSB biomass and specific growth rate were the highest at pH 7.[10]

Compared with the original condition (pH 6.8), specific growth rate and biomass production were increased by 100% and 27%, respectively, when the strain was cultured at pH 7. The treatment time could be reduced to five days for the maximum biomass production. Furthermore, COD removal rate was the highest when PSB was grown at pH 7 and it was higher than that of the original condition. It is shown that the change in pH (from 6.8 to 7) influenced the adaptation and metabolic activity of PSB.

Effect of light intensity on biomass production and COD removal

The effect of various light intensities on bacterial growth is shown in Figure 1(c). Bacterial growth began after an initial lag of 24 h and reached a stationary phase after three days of cultivation. At light intensity of 1000 lux, the specific growth rate was the lowest, resulting in the lowest biomass production (Table 1(c)). Although the COD consumed was the highest when light at 1000 lux was used, the biomass yield was the lowest compared with that obtained using other light intensity. In addition, the specific growth rate and biomass production under light at 1000 lux were less than those from the original condition which were illuminated at 2000 lux. These findings suggest that PSB used not only the substrate, but also certain light intensity for growth and metabolism. This is because light is an important factor for PSB growth since they convert light into chemical energy *via* photosynthesis.[5,24] Research have shown that PSB growth and COD removal were increased in the presence of light intensity from 2000 to 4000 lux, whereas further increases in light intensity resulted in a substantial decrease in the growth rate and COD reduction; thus, light is one of the limiting factors for PSB growth in a certain culture medium.[6,8,25] When light intensity was limited, bacterial growth would be low even in the high substrate environment. In contrast, light intensity at 4000 lux showed the highest growth rate and biomass production, resulting in the highest biomass yield and productivity. The increase in light intensity resulted in reducing the treatment time compared with the original condition, since high light intensity can provide more energy *via* photosynthesis, thus stimulating bacterial growth.[6] Based on the light at 4000 lux, the corresponding specific growth rate, biomass production, biomass yield and biomass productivity were increased by 100%, 29%, 167% and 200%, respectively, compared with the original condition. COD was decreased approximately 4250 mg/L and the treatment time was reduced from seven to three days. The short treatment time can minimize the operation cost and energy consumption in wastewater treatment.

Effect of additional carbon source on biomass production and COD removal

Glucose and sucrose are known to be the basic compounds which serve as carbon and energy sources for bacteria. Likewise, malic acid is a major carbon source for PSB. Therefore, these compounds were used in this study.

The addition of carbon sources significantly enhanced specific growth rate, biomass production, biomass yield and biomass productivity compared with the original condition (Table 1(d)). In the presence of glucose and sucrose, the growth rates reached the stationary phase within two days which reduced the treatment time significantly (Figure 1(d)). This is not surprising since sucrose is easily broken down to fructose and glucose in the metabolic pathway and the production of adenosine triphosphate (ATP) is generated by the catabolism of glucose *via* the glycolysis process.[26,27] Likewise, fructose can be converted to fructose-6-phosphate or glyceraldehyde-3-phosphate in the glycolysis to yield ATP. Moreover, the provision of building blocks for synthetic reactions is also associated with glucose dissimilation.[28] Therefore, bacteria could grow well when glucose and sucrose were supplemented. The addition of malic acid was found to significantly increase biomass accumulation by 279% relative to the original condition. This was because malic acid is a part of intermediates of the tricarboxylic acid cycle, which is the pathway to synthesize carbon skeleton and ATP. When malic acid is used as a carbon source, the enzymes involved in tricarboxylic acid cycle will take place to establish the cellular pools of reducing power for anabolic reactions and will involve in keeping the high ATP levels that makes malic acid a growth enhancer.[29,30] A recent study

Table 2. Crude protein content of *Rhodopseudomonas* sp. CSK01 biomass and other PSB.

Strains	Crude protein content (%)	Reference
Rhodopseudomonas sp. CSK01	60.1	This study
R. sphaeroides P47	66.6	[37]
R. gelatinosus	50	[38]
Rhodopseudomonas gelatinosa	62	[39]
Rhodopseudomonas gelatinosa	65	[40]

[10] has shown that the biomass production of photosynthetic bacterium, *Rhodopseudomonas faecalis*, was significantly increased when malic acid was supplemented in the medium. Biomass production in the wastewater supplemented with malic acid was greatly increased, but the specific growth rate was the lowest, suggesting that the addition of malic acid was found to exhibit long-term growth rate compared with the sugars. The research dealing with the use of glucose and malic acid as co-metabolism substrates for the degradation of o-chlorophenol by PSB *Rhodopseudomonas* sp. has shown that the growth rate of PSB in the presence of malic acid was lower than that in the presence of glucose, and the degradation period was extended when exposed to malic acid.[31] A similar study [32] has found that the use of glucose as co-substrate could reduce the lag phase of bacteria for waste removal. Additionally, purple PSB are able to decompose glucose in various growth conditions, whereas the utilization of malic acid is limited in some conditions.[33]

Interestingly, COD removal was significantly decreased in the presence of malic acid, whereas biomass accumulation was enhanced. To verify the results, this experiment was repeated and the results were similar. A plausible explanation for this phenomenon is that malic acid caused catabolite repression (CCR) because malic acid is considered as a preferred substrate for PSB. When PSB was cultured in the wastewater supplemented with malic acid which was preferentially utilized, the capability of PSB to consume the organic substances in wastewater was inhibited, resulting in the decline in COD assimilation. This hypothesis can be supported by [34]. CCR allows bacteria to assimilate a preferred carbon source when they are exposed to more than one carbohydrate. One of them is preferentially utilized, affecting the synthesis of catabolic enzymes or inhibiting the uptake of other carbon sources.[34] Recent research [35,36] have shown that malic acid is considered as the second preferred carbon source that causes CCR in *Bacillus subtilis*. Therefore, the addition of malic acid was found to enhance biomass accumulation instead of COD removal.

As mentioned above, the addition of glucose, sucrose and malic acid was not successful for wastewater treatment by this strain. Although the specific growth rate was increased and the treatment time was reduced when supplemented with sugars, the biomass production was not significantly different from the light experiments, and COD removal was lower. Moreover, CCR was shown to be initiated by malic acid, resulting in a low COD removal rate.

Determination of crude protein and amino acid composition

The protein content of *Rhodopseudomonas* sp. CSK01 grown in municipal wastewater under pH 7.0 and light at 4000 lux is presented in Table 2. It was comparable to the crude protein contents of *Rhodobacter sphaeroides* P47,[37] *Rhodocyclus gelatinosus*,[38] *R. gelatinosa*,[39] and *R. gelatinosa*[40] grown in medium containing pineapple peel waste, tuna condensate, soybean wastes and

Table 3. Amino acid composition of *Rhodopseudomonas* sp. CSK01 biomass and other PSB.

	Content (g/100 g dry cell)			
Amino acid	*Rhodopseudomonas* sp. CSK01	*R. sphaeroides* P47[a]	*R. gelatinosus*[b]	*R. gelatinosus* A1[b]
Histidine	4.36	0.96	1.13	1.01
Lysine	9.70	2.57	3.12	3.41
Phenylalanine	8.40	2.36	3.10	3.05
Leucine	8.38	3.90	5.41	5.28
Isoleucine	3.18	1.78	2.73	2.96
Methionine	1.00	1.47	1.89	1.71
Valine	2.80	2.68	3.42	3.75
Threonine	0.65	2.87	1.99	1.93
Cysteine	0.48	NA	NA	NA
Tryptophan	0.87	NA	NA	NA
Alanine	2.52	NA	NA	NA
Glycine	1.24	NA	NA	NA
Proline	1.38	NA	NA	NA
Glutamic acid	2.83	NA	NA	NA
Serine	0.93	NA	NA	NA
Tyrosine	5.73	NA	NA	NA
Aspartic acid	2.06	NA	NA	NA

[a]Data from [37]; [b]Data from [39]; NA, not analyzed.

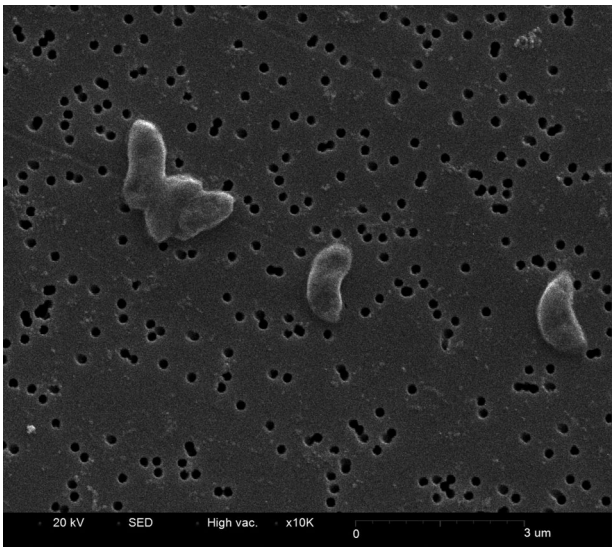

Figure 2. Scanning electron micrograph of *Rhodopseudomonas* sp. CSK01. Bar: 3 µm.

wheat bran, respectively. In addition to high protein content, the essential amino acid composition was comparable to other PSB as summarized in Table 3. In addition, the contents of histidine, lysine, phenylalanine and leucine were superior to those of the known SCP, *Chlorella vulgaris* and *Saccharomyces anomalus*, and the Food and Agriculture Organization guideline.[41,42] The microbial protein produced by *Rhodopseudomonas* sp. CSK01 contained all the essential amino acids required for animal feed, suggesting that this bacterium would be suitable as protein supplement and SCP for feedstuff.

In aquaculture, the size of the larval food is considered a problem because the larvae are tiny and they have small mouths that restrict the size of the food particles that can be ingested. As presented in Figure 2, the cell morphology of *Rhodopseudomonas* sp. CSK01 is vibrioid shaped, and the dimension of this strain is less than 3 µm which is suitable for utilizing as live food during the larval stage in aquaculture as well.

Conclusions

This study reveals that protein-rich biomass can be produced from municipal wastewater treatment. *Rhodopseudomonas* sp. CSK01 grown in wastewater under pH 7 and light at 4000 lux were optimal for municipal wastewater treatment and biomass production. In these conditions, COD removal reached 85% and the treatment time was three days with the maximum biomass of 6.2 g/L. The crude protein content of *Rhodopseudomonas* sp. CSK01 was 60.1%, containing all essential amino acids.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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