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Aquacultural Engineering 23 (2000) 281–293

aquacultural engineering

www.elsevier.nl/locate/aqua-online

Mass production of *Rhodopseudomonas palustris* as diet for aquaculture

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Received 23 November 1999

Abstract

Three different types of anaerobic fermentations were used for the mass production of the photosynthetic bacterium *Rhodopseudomonas palustris* as diet for aquaculture. The optimum agitation speed and malate concentration were 300 r.p.m. and 0.2% in the modified MYC medium, respectively. In batch fermentations of *R. palustris*, the maximum number of viable cells was 1.1×10^{10} c.f.u. ml^{-1} with 2.65 g l^{-1} of DCW, and the maximum specific growth rate and biomass productivity were estimated to be 0.12 h^{-1} and 55 mg l^{-1} h^{-1}, respectively. Crude protein content of *R. palustris* was about 72–74%. The composition of stearic acid and oleic acid of *R. palustris* was superior to those of *Chlorella* and yeasts, while that of other fatty acids tested was not. The amino acid profiles of the protein hydrolysate compared favorably with Food Agricultural Organization (FAO) guidelines. The biomass productivities from fed-batch experiments were found to be 50, 47 and 49 mg l^{-1} h^{-1} for linear, exponential, and sigmoidal feeding strategy, respectively. The maximum biomass productivity was found to be 112 mg l^{-1} h^{-1} in chemostat. Compared to growth in batch cultures, continuous fermentation yielded two times higher biomass productivity.

Keywords: Biomass productivity; *Rhodopseudomonas palustris*; Single cell protein; Nutritive value

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0144-8609/00/$ - see front matter
PII: S0144-8609(00)00057-1

1. Introduction

It is becoming increasingly evident that the development of low-cost, high-quality protein feed is crucial for the future success of aquaculture industry (Rumsey, 1978). As single cell proteins (SCP), microalgae have been used as an essential food for the larval stages of fish and shellfish (Benemann, 1992) and yeasts have been considered as algal substitute for several species of filter feeders (Epifanio, 1979; Fukusho, 1980; Coutteau et al., 1990; Kim et al., 1998), but the industrial production of SCP from microalgae and yeasts has not been extensive so far.

Compared to microalgae and yeasts, photosynthetic bacteria have some advantages as a diet for aquaculture. They are available as a by-product from agricultural wastes (Shipman et al., 1975), have more digestible bacterial cell wall, and are rich in protein, carotenoids, biological cofactors, and vitamins (Kobayashi and Kurata, 1978). It was reported that addition of photosynthetic bacteria as food source stimulated the growth of zooplankton much more than green algae did, and are very useful for growth of brine shrimp (Kobayashi, 1995). Further, photosynthetic bacteria were also identified as prey material for that fish fry (loach, goldfish, crap, ark shell, sweetfish, etc.) soon after hatching, resulting in an increase in weight and survival rate of more than twofold within 2–4 weeks after hatching. The rate of survival increased markedly and almost no mortality was observed when 0.1% live cells of photosynthetic bacteria were added to the formula feed given to the fry of crucian crap soon after hatching. It was also reported that prawn culture tanks were frequently affected by gill disease causing great economic damage, but this was completely prevented by supplementing the tanks with photosynthetic bacteria. Such effects have also been displayed in suppression of virus diseases noted on others including shellfish (Okamoto et al., 1988; Hirotai et al., 1991).

The mass production of photosynthetic bacteria as SCP seems to be an interesting solution to the growing problem of protein-rich supplement for aquaculture. To be economically feasible, it is necessary to engineer optimum culture conditions for maximum biomass productivity. Most studies concerning the mass cultivation of photosynthetic bacteria relate to batch-cultures (Driessens et al., 1987). Thus, optimum culture conditions and nutritive value were investigated in anaerobic batch culture and biomass productivities were estimated in three different types of anaerobic fermentations.

2. Materials and methods

2.1. Microorganism and media

The strain used in this study was *Rhodopseudomonas palustris* isolated by Kim et al. (1999) from photosynthetic sludge because of its high denitrifying capacity. Stock cultures were maintained by monthly transfer into the modified MYC (Table 1) agar plate. The liquid modified MYC medium was used for seed culture. In batch fermentations, the modified MYC medium containing 0.1 or 0.2 or 0.3% malate was used for optimization.

2.2. Cultivation

2.2.1. Seed culture

An isolated colony was transferred with a platinum needle into the screw-cap tube under aseptic conditions. The tube was completely filled with the modified MYC medium and closed tightly, then incubated at 31°C and rotated at 100 r.p.m. under incandescent illumination at 5000 lux. The cells were harvested at the end of the exponential growth phase, and 10% of those cells were used as an inoculum for the flask culture. The flask culture was carried out in a 500 ml shake flask with 200 ml working volume. Hungate technique was used to create an anaerobic condition inside the flask, in which O_2-free argon gas was flushed (Bryant, 1972).

2.2.2. Batch fermentation

Batch fermentations were conducted using 1 l- and 5 l-fermenter with the working volume of 700 ml and 3500 ml, respectively at 31°C and 5000 lux illumination with external incandescent bulbs. The pH was controlled at 5.5 by using 2N-HCl and 2N-NaOH in the 1 l-fermenter and 3N-HCl and 3N-NaOH in 5 l-fermenter, and foam was controlled using 10% antifoam DB-110A. An anaerobic condition inside fermenter was created by flushing O_2-free argon gas until concentration of dissolved oxygen dropped to zero. Ten percent of inoculum from the flask culture was used as seed culture. In order to determine optimum agitation speed and malate concentration batch fermentations were conducted at different conditions of agitation speeds (200, 250, 300 and 350 r.p.m.) and malate concentrations (0.1, 0.2 and 0.3%), respectively. A six flat-bladed impeller (diameter, 80 mm) was used for agitation.

Table 1
Composition of the modified MYC medium

Component	Amount (l^{-1})
Malate (g)	1
Casamino acid (g)	2
Yeast extract (g)	3
Trace element solution† (ml)	1
†*Trace element solution*	
$FeSO_4 \cdot 7H_2O$ (g)	3
H_3BO_3 (mg)	10
$Na_2MoO_4 \cdot 2H_2O$ (mg)	10
$MnSO_4 \cdot H_2O$ (mg)	20
$CuSO_4 \cdot 5H_2O$ (mg)	10
$ZnSO_4$ (mg)	10
Ethylenediamine-Tetraacetic acid (mg)	500

2.2.3. Fed-batch fermentation

Fed-batch fermentations were conducted in 1 l-fermenter under the same conditions as the batch fermentation. After one batch culture, fermentation broth was pumped out of fermenter vessel leaving 10% volume for inoculum when the batch culture reached the stationary phase, and then the fed-batch fermentation started with feeding. Feed was pumped into the fermenter by a peristaltic pump by three feeding pattern (linear, exponential, and sigmoidal). The pH, temperature, and foam were controlled in the same way as the batch fermentation. In order to maintain anaerobic conditions inside fermenter the feed medium was flushed with O_2-free argon gas before being fed into fermenter. The agitation speed was controlled in the range of 100–300 r.p.m.

2.3. Continuous fermentation

A continuous fermentation was conducted in the 1 l-fermenter after batch fermentation under the same conditions as the batch fermentation. The continuous fermentation was initiated at the stationary phase of actively growing batch culture. The pH, temperature, foam, and anaerobic condition in the chemostat were controlled in the same way as fed-batch fermentation. Agitation rate was 300 r.p.m. The dilution rate was low at first and then increased stepwise from 0.01 to 0.12 h^{-1} using a two-way Masterflex peristaltic pump. A steady state was considered to be reached at each dilution rate after five fermenter volumes had passed through the system.

2.4. Assays

Samples from the fermenter were analyzed to measure the concentration of photosynthetic bacteria. The cell concentration was measured spectrophotometrically at 660 nm. Samples were diluted in order to confine the absorption readings to the range 0.1–0.7 optical densities (OD), as required by the Lambert-Beer law. To relate the measured OD to the dry-cell weight (DCW) of photosynthetic bacteria, several samples were taken at different times over the course of fermentations. The DCW was determined by weighing the cell pellet after being dried in an oven at 100°C for 12 h. The cell pellet was prepared by centrifuging a 5 ml sample of fermentation broth at 5000 r.p.m. for 10 min, and then by decanting the supernatant after washing twice with distilled water. A linear correlation between DCW and OD was obtained. The number of viable cells was calculated by plate counting.

Water content was analyzed by the atmospheric heating method, crude protein by the Kjeldahl method, lipid by Soxhlet method, and ash by the direct ashing method (AOAC, 1984). Amino acid content was analyzed with an amino-acid autoanalyzer (Biochrome 202). Composition of fatty acids was analyzed by gas chromatography (HP 6890) using a HP20M (0.25×30 m) column. Carrier gas was Helium at 10 p.s.i., and the temperatures of injector and flame ionized detector were 250 and 270°C, respectively. The column temperature was 150°C at the beginning, and increased from 150 to 230°C at a rate of 2°C min^{-1}.

3. Results and discussion

3.1. Batch fermentation

Batch fermentations were first executed in a 5 l fermenter at 300 r.p.m. under the optimum growth conditions determined in our previous study (Kim et al., 1999). The growth rate of *R. palustris* slowed down after 25 h and reached the stationary phase after 80 h. This growth retardation in culture of photosynthetic bacteria has also been reported by Sawada and Rogers (1977). The maximum growth rate was calculated to be 0.068 h^{-1}, and it is lower than that (0.095 h^{-1}) obtained from the seed culture of 500 ml flask. It is postulated that this phenomenon is probably due to light transfer limitation at higher cell mass concentration. To reduce this light transfer limitation, a 1 l fermenter was used and compared to the 1 l- and 5 l-fermentations is shown in Fig. 1. All growth values indicate that less light transfer limitation was observed in the 1 l-fermenter. This reflects that different geometry of fermenter vessel changed light transfer. Hereafter, all fermentations were conducted in the 1 l-fermenter.

Four different agitation speeds were tested in order to determine optimum agitation speed. As seen in Fig. 1, the growth of *R. palustris* increased when the agitation speed rose up to 300 r.p.m. It is thought that effects of mixing and light transfer increased. But, the cell growth decreased above 300 r.p.m. probably due to shear stress. Optimum malate concentration was determined to be 0.2% (Fig. 1). At a malate concentration above 0.2% the biomass yield decreased. The metabolic pathways of *R. palustris* may be changed at higher concentrations of malate. A similar result was found in the study of photosynthetic bacteria, *Rhodospirillum rubrum* (Gest et al., 1950).

Under the optimum conditions obtained from the above experiments, the batch fermentation was executed in a 1 l-fermenter. The maximum number of viable cells was 1.1×10^{10} c.f.u. ml^{-1} with 2.65 g l^{-1} of DCW, and the maximum specific growth rate and biomass productivity were estimated to be 0.12 h^{-1} and 55 mg l^{-1} h^{-1}, respectively.

The nutritive value of *R. palustris* as a diet was investigated. Crude protein content was about 72–74% (Table 2). This protein content was higher than most photosynthetic bacteria (61.0%), *Chlorella* (55.5%) and yeast cells (50.5%) (Kobayashi and Kurata, 1978). No significant differences among the cell components and the fatty acid composition of *R. palustris* were found between both cell culture media and the growth phases ($P < 0.95$). The Bonferroni multiple comparison was used to identify differences significant at the 95% confidence level. It has well known that highly unsaturated fatty acids are largely responsible for membrane fluidity, which is critical during tissue differentiation. They also act as a major energy source in fish larvae (Watanabe, 1982). Studies on the essentiality of unsaturated fatty acids in the diets of fish (Bell et al., 1986; Sargent et al., 1989) have demonstrated that marine species of finfish require *n*-3 highly unsaturated fatty acids for normal growth. The composition of stearic acid (18:0) and oleic acid (18:1) of *R. palustris* was superior to that of *Chlorella* (Ishida et al., 1998) and yeasts (Daum et al., 1999), while that of other fatty acids tested was not.

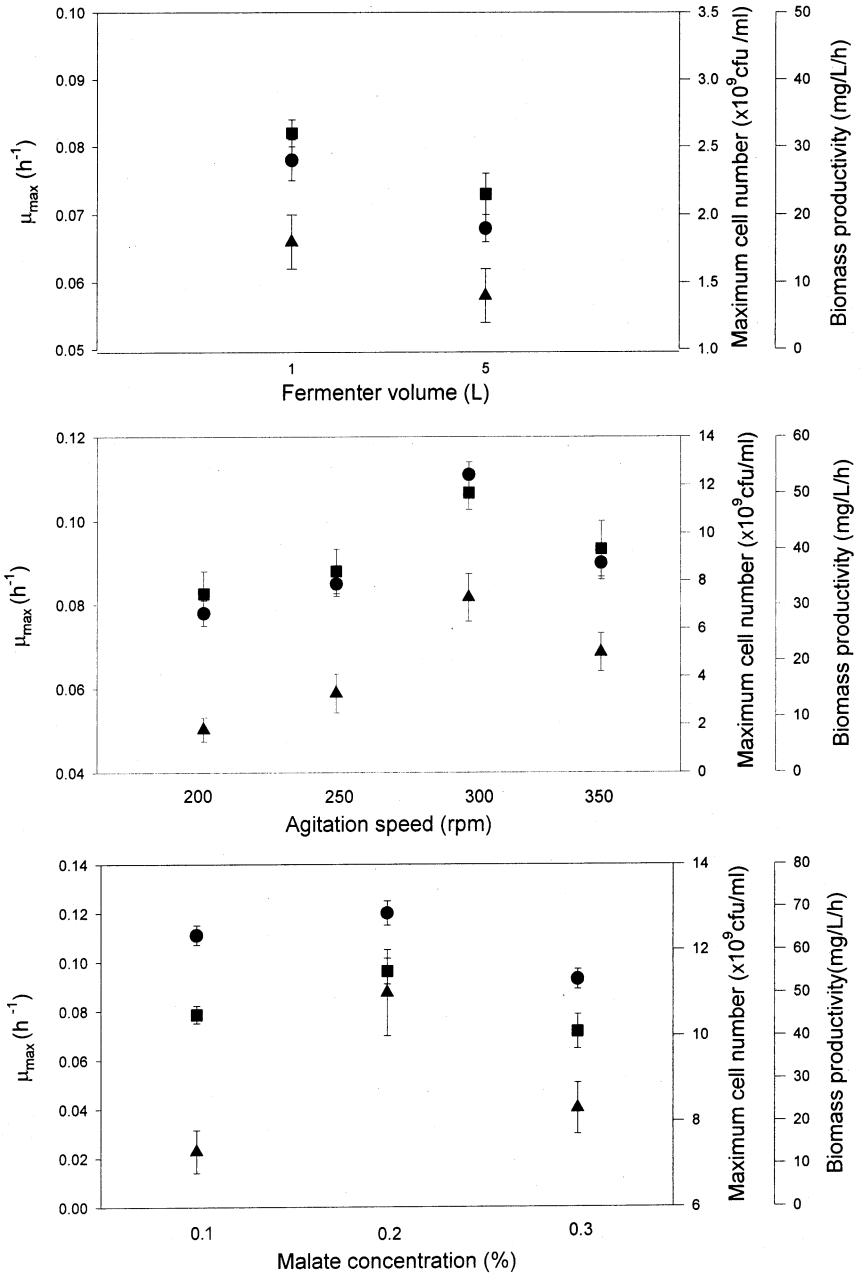

Fig. 1. μ_{max} (●), maximum cell number (▲), and biomass productivity (■) under different conditions. The results were obtained from batch fermentations of *R. palustris*. Error bars, ±1.0 SD.

Table 2
Cell components and fatty acid composition of *R. palustris* in anaerobic batch fermentations

Medium growth phase		MYC with 0.2% malate[a]		Modified MYC with 0.2% malate[b]	
		Late-log	Stationary	Late-log	Stationary
Cell components[c] (%)	Moisture	1.7 ± 0.4	0.9 ± 0.5	1.9 ± 0.4	1.1 ± 0.4
	Protein	73.4 ± 0.1	72.3 ± 0.1	73.9 ± 0.1	72.4 ± 0.1
	Lipid	1.0 ± 0.4	1.9 ± 0.3	2.4 ± 0.3	1.5 ± 0.3
	Ash	6.1 ± 0.1	5.9 ± 0.1	6.7 ± 0.2	6.1 ± 0.2
Fatty acid[c] (% of total lipids)	14:1	ND	ND	0.6	ND
	16:0	13.8 ± 0.3	13.6 ± 0.3	12.6 ± 0.4	13.5 ± 0.2
	16:1	1.3 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
	18:0	12.5 ± 0.3	11.9 ± 0.2	12.5 ± 0.3	11.8 ± 0.3
	18:1	71.5 ± 0.1	71.4 ± 0.1	71.3 ± 0.1	71.5 ± 0.1
	20:0	ND	ND	ND	ND
	20:1	0.9 ± 0.3	1.2 ± 0.2	1.6 ± 0.3	1.9 ± 0.3
	20:5*n*-3	ND	0.6	ND	ND
	24:0	ND	0.3	ND	ND

[a] Medium composition is 0.2% malate, 0.2% casamino acid, and 0.3% yeast extract.
[b] Medium composition is 0.2% malate, 0.2% casamino acid, 0.3% yeast extract, and 1ml trace element solution (listed in Table 1).
[c] Average of two replicates prepared from each sample (mean ± SD). The Bonferroni multiple comparison test was used. ND: Not detected.

The composition of amino acid of *R. palustris* is shown in Table 3. In addition to the high protein content, the amino-acid profiles of the protein hydrolysate compared favorably with the Food Agricultural Organization (FAO) guideline (FAO, 1980). The contents of histidine, arginine and tyrosine were better than those of three other microorganisms in Table 3. Many fish require high levels of good quality protein in their diets, but the use of dietary protein for growth depends ultimately upon the availability of a suitable balance of amino acids (Barroso et al., 1999). Compared to the experimental diets used for rainbow-trout (Barroso et al., 1999), the amino-acid composition of *R. palustris* was almost the same in quantity. Methionine has known to be the limiting amino acid in single-cell protein (Shipman et al., 1975), but our results show that *R. palustris* clearly had almost the same methionine quantity as called for by the FAO guideline.

3.2. Fed-batch fermentation

The fed-batch fermentations of *R. palustris* were executed with three different types of feeding strategies. The feeding patterns are linear, exponential, and sigmoidal which are shown in Fig. 2B. For each feeding pattern, a total volume of 800 ml was provided within 50 h, which was set according to the result of batch

Table 3
Amino acid composition of different types of single cell protein

Amino acid (g/100 g protein)	Protein source				
	Rhodopseudomonas palustris	FAO guideline[a]	*Rhodobacter*[b] *capsulata*	*Chlorella vulgaris*[b]	*Saccharomyces*[b] *anomalus*
Lysine	5.13	4.20	4.69	4.88	7.44
Histidine	2.71		2.05	1.90	1.78
Arginine	6.51		5.48	5.84	4.95
Aspartic acid	6.90		7.48	8.54	6.16
Threonine	3.82	2.80	4.42	4.10	5.24
Serine	3.23		2.75	3.81	5.44
Glutamic acid	8.17		8.75	8.32	12.3
Proline	3.64		4.67	3.81	3.50
Glycine	4.11		3.95	4.10	4.32
Alanine	6.36		7.62	5.36	5.66
Valine	4.51	4.20	5.75	5.44	6.33
Methionine	2.14	2.20	2.59	0.48	1.00
Isoleucine	3.40	4.20	4.40	4.39	5.20
Leucine	6.18	4.80	7.50	8.03	7.00
Tyrosine	2.97		2.80	1.73	2.57
Phenylalanine	3.67	2.80	4.33	4.77	4.35

[a] Data from FAO (1980).
[b] Data from Kobayashi and Kurata (1978).

fermentation. Feeding rate was changed at each datum point in Fig. 2B for each feeding strategy. The profile of exponential feeding to the culture shows little fluctuation of cell mass production, but the lowest yield of biomass was produced (Fig. 2A). This seems that this feeding strategy did not match the growth rate of *R. palustris*. The profiles of linear feeding and sigmoidal feeding show some fluctuation probably due to dilution. The best result was obtained with linear feeding strategy, but the difference among the three biomass yields was slight.

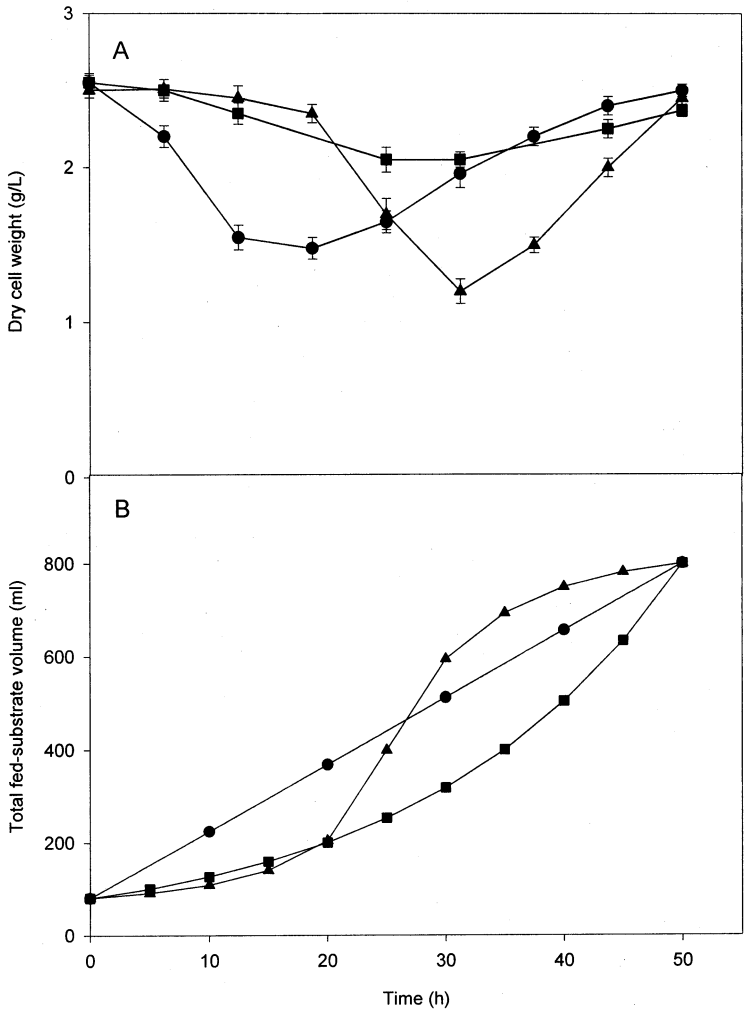

Fig. 2. Profiles of biomass yield (A) and total fed-substrate volume (B) along fermentation time in fed-batch fermentations of *R. palustris* with different feeding strategies (Linear, –●–; Exponential, –■–; Sigmoidal, –▲–). Error bars, ±1.0 SD.

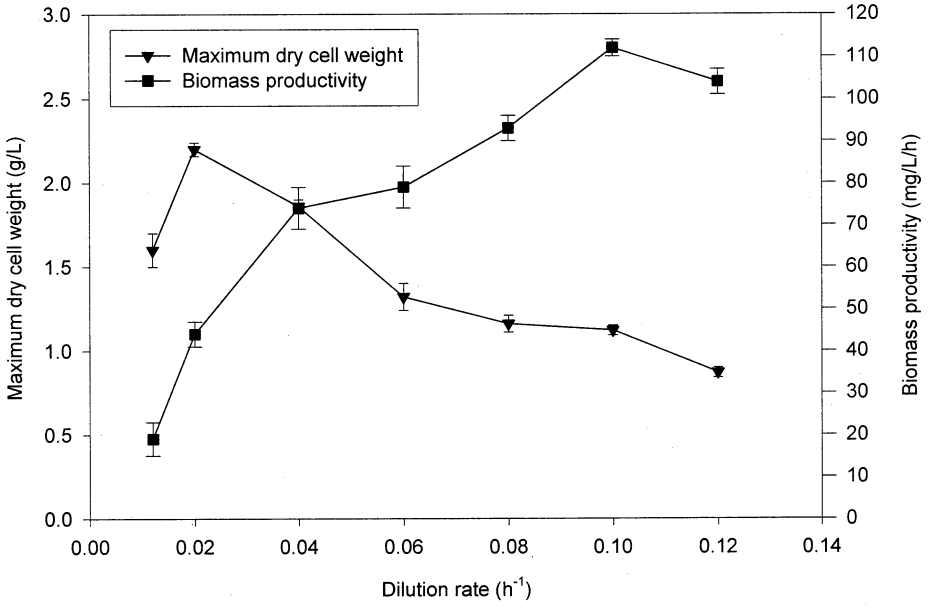

Fig. 3. A continuous fermentation of *R. palustris* on the modified MYC medium containing 0.2% malate. Error bars, ± 1.0 SD.

The fed-batch experiments of *R. palustris* with three different strategies produced 2.50, 2.37, and 2.45 g l^{-1} of DCW for linear, exponential, and sigmoidal feeding, respectively. It has known that the fed-batch fermentation is necessary to avoid effects as substrate inhibition and catabolic repression (Mignone and Avignone Rossa, 1993). To reduce these effects, continuous addition of fresh medium should be operated and this results in increase of feed blending since the substrate is rapidly depleted in the fermenter (Bajpai and Reuss, 1982). But, no improvement was found by fed-batch experiments in our study. The biomass productivities were 50, 47 and 49 mg l^{-1} h^{-1} for linear, exponential, and sigmoidal feeding, respectively.

3.3. Continuous fermentation

Continuous fermentations were executed on the modified MYC medium containing 0.2% malate under the optimum conditions. As seen in Fig. 3, the maximum biomass of 2.2 g l^{-1} was achieved at a dilution rate of 0.02 h^{-1}, whereas it decreased up to 0.12 h^{-1} with increasing dilution rate. Chemostat experiments were executed up to this dilution since wash-out phenomenon may occur. The maximum biomass productivity was found to be 112 mg l^{-1} h^{-1} at dilution rate of 0.10 h^{-1}. The dilution rate that resulted in the maximum productivity was not the same dilution rate that produced maximum biomass yield. Consequently, a compromise must be made.

Table 4
Maximum biomass productivities from three different types of fermentations

Fermentation type	Maximum biomass productivity (mg/l per h)	Biomass at maximum biomass productivity (g/l)
Batch	55	2.65
Fed-batch		
Linear	50	2.50
Exponential	47	2.37
Sigmoidal	49	2.45
Continuous	112	1.12

The maximum biomass productivities obtained from three different types of anaerobic fermentations are shown in Table 4. The best result was 112 mg l^{-1} h^{-1} obtained from the continuous fermentation. This result is not surprising since high cell density culture is difficult under anaerobic conditions with illumination because of light transfer limitation (Watanabe et al., 1998). This result was somewhat lower, compared to that (165 mg l^{-1} h^{-1}) obtained by using *Rhodopseudomonas gelatinosa* cultured on 3% wheat bran infusion culture medium (Shipman et al., 1975). The differences may be due to different species of photosynthetic bacteria, type of substrate, substrate concentration, and limitation of light supply, etc. Compared to growth in batch cultures, continuous fermentation yielded two times higher biomass productivity.

4. Conclusions

The mass production of photosynthetic bacteria as SCP seems to be an interesting solution to the growing problem of protein-rich supplement for aquaculture. Three different types of anaerobic fermentations were executed for the mass production of photosynthetic bacteria, *Rhodopseudomonas palustris*, as diet for aquaculture. The denitrifying strain, *Rhodopseudomonas palustris*, was used in this study. The optimum agitation speed and malate concentration were 300 r.p.m. and 0.2%, respectively. In batch fermentations, the maximum number of viable cells was 1.1×10^{10} c.f.u. ml^{-1} with 2.65 g l^{-1} of DCW, and the maximum specific growth rate and biomass productivity were estimated to be 0.12 h^{-1} and 55 mg l^{-1} h^{-1}, respectively.

Crude protein content of *R. palustris* was about 72–74%. No significant differences of the cell components and fatty acid composition of *R. palustris* were found between both cell culture media and the growth phases ($P < 0.95$). The composition of stearic acid (18:0) and oleic acid (18:1) of *R. palustris* was superior to those of *Chlorella* and yeast, while that of other fatty acids tested was not. The amino-acid profiles of the protein hydrolysate compared favorably with the Food Agricultural Organization (FAO) guideline.

The fed-batch experiments of *R. palustris* with three different strategies produced 2.50, 2.37, and 2.45 g l^{-1} of DCW for linear, exponential, and sigmoidal feeding, respectively. The biomass productivities were 50, 47 and 49 mg l^{-1} h^{-1} for linear, exponential, and sigmoidal feeding, respectively. The maximum biomass of 2.2 g l^{-1} was achieved at a dilution rate of 0.02 h^{-1} in continuous fermentations. The maximum biomass productivity was found to be 112 mg l^{-1} h^{-1}. This result is not surprising since high cell density culture is difficult under anaerobic conditions with illumination because of light transfer limitation. Compared to growth in batch cultures, continuous fermentation yielded two times higher biomass productivity.

The estimated cost of *R. palustris* production based on the 1 l-batch fermentation is projected to be US$79/kg dry cells, which seems to be rather higher than that of algae (Duerr et al., 1998). The study of mass culture in a large-scale process, in which light transfer limitation in high cell density culture is overcome, is needed in order to reduce the production costs while maintaining reliability. Consequently the production of *R. palustris* for use as aquaculture feed is a viable alternative for producing SCP.

Acknowledgements

This work was supported by the Korea Science Engineering Foundation (KOSEF) (Grant No. 97K3-1506-01-03-3) through the Research Center for Ocean Industrial Development at Pukyong National University.

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