Pure & Appl. Chem., Vol. 69, No. 11, pp. 2439–2445, 1997.
Printed in Great Britain.

Utilization and treatment of tuna condensate by photosynthetic bacteria

Poonsuk Prasertsan[a] Marisa Jaturapornpipat[a] and Chairat Siripatana[a]

[a] *Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hatyai, 90110 THAILAND*

Abstract: Tuna condensate, the effluent discharged from a steam cooker after precooking tuna at 100°C for about 1 h, is a major source of high organic loading of the wastewater in seafood processing factories. To reduce its organic content and to prevent environmental problems, it is utilized as a substrate for single cell protein production.

Factors affecting the growth of *Rhodocyclus gelatinosus* R7 and the reduction of organic matter (represented as COD) was further investigated in the diluted tuna condensate (COD=20,000 mgl^{-1}) incubated under anaerobic-light (3000 Lux) condition. Supplementation of yeast extract in the medium significantly increased the biomass and the pigment content of *R. gelatinosus* and its optimum concentration was 3.0 gl^{-1}. The highest biomass was 6.24 gl^{-1} with COD removal of 38%. Without yeast extract, the corresponding values were 3.96 gl^{-1} and 24%, respectively. The optimum concentration of $MgCl_2$ for cell growth was found to be 10 mM but the pigment content decreased significantly as the concentrations of $MgCl_2$ higher than 5 mM. Addition of cobalt chloride (1-160 μM) and ferrous sulphate (10-120 μM) gave results with no significant difference from the control. Controlling the pH at 7.0 during the cultivation in a 2 l fermenter exhibited higher values of biomass and COD reduction than without pH control. With the added yeast extract (3 gl^{-1}) and $MgCl_2$ (5 mM) as well as controlling the pH at 7.0, the highest biomass obtained was 8.14 gl^{-1} with the COD removal of 59%. Four folds increase of biomass (to 12.27 gl^{-1} compared to 3.96 gl) was achieved by repeated addition of the fresh tuna condensate (COD=60,000 mgl^{-1}) every 40 h. The cell contained 56% protein, 2.21 mg carotenoid and 22.52 mg bacteriochlorophyll/g dry cell weight. The COD removals were 66% and 46% after 5 and 7 days cultivation, respectively.

Keyword : tuna condensate, photosynthetic bacteria, *Rhodocyclus gelatinosus,* waste utilization, waste treatment

INTRODUCTION

Seafood industry is one of the three major agro-industries in Southern Thailand with 40 plants in operation. Thailand is the world leader in canned tuna exports. There are large quantities of waste generated during the process which may lead to environment problems. Tuna condensate, the effluent discharged from a steam cooker after precooking tuna at 100°C for about an hour, is normally discharged into the wastewater treatment system although it is a good source of nutrients. Too high organic loading could cause some problems such as the occurrence of a red color of wastewater in the pond which was found to be the photopigment of photosynthetic bacteria (ref. 1).

The above problem could be mitigated by separating the high organic content sources; the tuna condensate. The condensate could be used as culture medium for the four isolated strains of *Rhodocyclus gelatinosus* (ref. 2). Photosynthetic bacteria have been employed for the treatment of wastewater and single cell protein production (ref. 3,4,5). They are rich in protein (40-69% w/w), carotenoid (0.09 to 0.80 mg per g dry cell weight, DCW), vitamin B_{12} (30-79 mg per kg DCW) and essential amino acids (ref. 6). Cultivation of photosynthetic bacteria in tuna condensate therefore could have a two-fold benefit: production of single cell protein and reduction of organic matter in the wastewater.

Optimization for growth of *R. gelatinosus* R7 and treatment of tuna condensate has been studied (ref. 2). This work is a continuation of the investigation on the nutritional requirement, effects of controlling pH and repeated addition of the fresh tuna condensate to improve biomass production and increase COD reduction.

MATERIALS AND METHODS

Substrate

Tuna condensate was kindly provided by Tropical Canning Co., Ltd., Hat yai, Thailand.

Microorganism

R. gelatinosus R7, the strain isolated from the seafood processing wastewater(ref. 7) was used. The culture was maintained on G5 agar slant and kept in a refrigerator.

Medium

G5 medium used for the preparation of the inoculum contained (gl^{-1}) : peptone, 5.0; yeast extract, 5.0; L-glutamic acid, 4.0; KH_2PO_4, 0.12 and K_2HPO_4, 0.18 (ref. 8). The initial pH of the medium was adjusted to pH 7.0 using 5 M NaOH.

Tuna condensate medium was prepared by diluting the tuna condensate with distilled water to reach the COD value of 20,000 mgl^{-1}. The pH was adjusted to 7.0.

Analytical Methods

The growth of the culture was measured turbidometrically at 680 nm and converted to dry cell weight (DCW) using a calibration curve. Cell pigments (carotenoids and bacteriochlorophyll) were determined following the methods described by Hirayama (ref. 9). Chemical oxygen demand (COD), total Kjeldahl nitrogen, oil & grease, total solids, suspended solids, total phosphorus and chloride were analysed according to the APHA, AWWA and WPCF (ref. 10). Protein was measured using the Kjeldahl method (ref. 11). Magnesium and iron were determined using atomic absorption spectroscopy (ref. 12).

Characteristics of Tuna Condensate

Tuna condensate was analysed for COD, total Kjeldahl nitrogen, oil & grease, total solids, suspended solids, total phosphorus, chloride, magnesium and iron according to the procedure mentioned above.

Optimization Procedure

The inoculum of *R. gelatinosus* R7 was prepared in G5 medium (ref. 2) and inoculated (10% v/v) into the 200 ml tuna condensate medium. The culture was incubated under anaerobic-light (3000 Lux) conditions. Samples were taken every 24 h, when pH, biomass, COD and pigment contents were measured. The effect of yeast extract was studied at concentrations of 0.1, 0.5, 1.0 and 3.0 gl^{-1}. The metal ions investigated were $CoCl_2.6H_2O$ (10-160 μM), $MgCl_2.6H_2O$ (0.5-30 mM) and $FeSO_4.7H_2O$ (10-120 μM).

The effect of pH on growth and waste treatment was investigated by controlling the pH at 7.0, using 1 N HCl, during the cultivation under anaerobic-light conditions with an agitation speed of 100 rpm. The experiment was conducted in a 2 l fermenter containing 1.5 l working volume of the tuna condensate medium supplemented with yeast extract. The medium was flushed with nitrogen gas at a pressure of 69 kP for 3 h to remove air before the inoculation. Batch culture in the presence of selected metal ion(s) was carried out for comparison. The effect of repeated addition of the fresh tuna condensate (COD = 60,000 mgl^{-1}) was conducted by adding the condensate every 40 h.

RESULTS AND DISCUSSION

The characteristics of tuna condensate are presented in Table 1. The pH of 6.07 was slightly lower than the optimal pH range (6.5-7.5) for photosynthetic bacteria and the pH optimum (7.0) of *R. gelatinosus* R7. Therefore, the pH of the tuna condensate medium was adjusted to pH 7.0. The COD of this batch (74,000 mgl^{-1}) was approximately half of that reported earlier (ref. 2) and the oil & grease content was also lower. The variation was due to the difference in tuna species, fish sizes, catching area and season. The other parameters, however, did not differ very much from those reported previously except for the high content of magnesium and chloride.

The effect of yeast extract addition (0-5 gl^{-1}) on growth, COD removal and pigment synthesis of *R. gelatinosus* R7 in tuna condensate medium is illustrated in Table 2. Results indicated that biomass, COD removal and cellular pigment content increased as the concentrations of yeast extract increased. The optimum concentration of yeast extract was 3 gl^{-1} which gave the highest biomass of 6.24 gl^{-1} with a maximal COD removal of 38%. The highest carotenoid and bacteriochlorophyll contents were 3.09 and 26.35 mg g^{-1} DCW, respectively. The optimum concentration of yeast extract noted during this work was higher than that (2 gl^{-1}) required for the growth of *Rhodopseudomonas capsulata* with the maximum biomass of 1.18 gl^{-1} (ref. 4). It should be noted that in both cases, the highest biomass was obtained at the highest concentration of yeast extract tested.

The influence of yeast extract on the growth and pigment synthesis of *R. gelatinosus* R7 was due to the presence of nitrogen, carbohydrate, and various growth factors including biotin and thiamine (ref. 13). A positive requirement for biotin and thiamine during growth of *R. gelatinosus* R7 has been reported previously (ref. 7). Biotin was needed for the activity of 5-aminolevulinic acid (ALA) synthetase in the formation of ALA which is the intermediate of bacteriochlorophyll synthesis. Thiamine, on the other hand, is the substrate of the coenzyme thiamine pyrophosphate that converts alpha-oxaglutarate to a C4-intermediate which combines with glycine to produce porphyrin, the precursor for the synthesis of bacteriochlorophyll, heme and vitamin B_{12} (ref. 14). Histidine and glutamic acid in the yeast extract were found to enhance the assimilation of propionate by *Rhodopseudomonas capsulata* (ref. 15).

Table 1: Characteristics of tuna condensate from a seafood processing plant

Parameter*	Mean
pH	6.07
COD	73,617
Total Kjeldahl nitrogen	6,644
Oil & Grease	668,450
Total solids	6,541
Suspended solids	586
Total phosphorus	95
Magnesium	5,80
Iron	2,625
Chloride	19,656

Table 2: Effect of yeast extract on growth, pigment synthesis and COD removal from cultivation of *R. gelatinosus* R7 in tuna condensate medium

Yeast extract conc. (gl^{-1})	Biomass (gl^{-1})	Pigment (mg g^{-1} DCW)		COD removal (%)
		carotenoid	Bacteriochloro-phyll	
0	3.96	2.13	20.76	24
0.1	4.11	2.04	21.46	26
0.5	5.02	2.17	23.33	27
1.0	5.69	2.24	23.02	36
3.0	6.24	3.9	26.35	38

The effects of the concentrations of Co^{2+}, Fe^{2+}, Mg^{2+} on the growth, COD removal and pigment content of *R. gelatinosus* R7 cultivated in the tuna condensate medium with yeast extract (3 gl^{-1}) were also investigated. The growth decreased at $CoCl_2$ concentrations higher than 1 μM giving a biomass yield of 6.14 gl^{-1} and COD removal of 37% (Fig. 1). Cobalt (10-160 μM) inhibited the cell growth with the biomass decreasing as the concentration of cobalt increased. This also occurred during cultivation of *Rhodospirillum rubrum* G-9 BM in Hoshino's basal medium with the same range of $CoCl_2$ concentrations (ref. 16). $CoCl_2$ (4 μM) together with yeast extract was reported to increase the growth and vitamin B_{12} content of *Rhodopseudomonas sphaeroides* S grown in a propionate medium (ref. 17). Supplementation of 4 μM

$CoCl_2$ also enhanced the production of vitamin B_{12} by *Rhodopseudomonas sphaeroides* 8253 in GM medium by approximately 2.5 and 12-fold under aerobic-dark and anaerobic-light conditions, respectively (ref. 18). The higher cellular content of both carotenoid (2.39 mg g^{-1}) and bacteriochlorophyll (21.29 mg g^{-1}) than the control was observed at 10 μM $CoCl_2$ after 5 days cultivation, although these values were not particularly different from those obtained at other cobalt ion concentrations. The pigment content of this strain was higher than that of *Rhodopseudomonas sphaeroides* S grown in pineapple juice medium with the addition of 1.2 mgl^{-1} (20.37 μM) of cobalt chloride (ref.19). A high concentration (1.9 mgl^{-1}) of cobalt chloride caused a reduction in vitamin B_{12} synthesis.

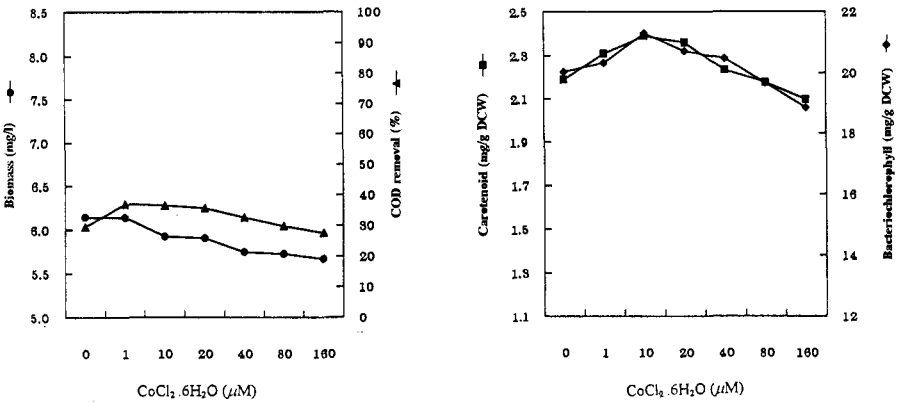

Fig.1 Effect of the concentrations of cobalt chloride on growth, COD removal and pigment synthesis of *Rhodocyclus gelatinosus* R7 grown in tuna condensate medium supplemented with yeast extract (3g/l) under anaerobic-light condition at room temperature.

The influence of ferrous sulphate is shown in Fig. 2. The addition of 10 μM $FeSO_4$ slightly increased both the biomass (6.34 gl^{-1}) and the COD removal (44%), after 5 days cultivation, but decreased at higher concentrations. This concentration (10 μM) was lower than that (36 μM $FeSO_4$) required for the growth of *Rhodospirillum rubrum* G-9 MB (ref. 16). Ferric citrate was the source of iron for growth and pigment synthesis of *Rhodopseudomonas sphaeroides* S and its optimum concentration was 4 mgl^{-1} (ref. 19). The carotenoid content of *R. gelatinosus* R7 remained almost constant regardless of the quantities of $FeSO_4$ added except at 60 μM $FeSO_4$. The bacteriochlorophyll content, however, increased slightly as the concentrations of $FeSO_4$ increased. High iron concentrations inhibit growth and pigment synthesis of photosynthetic bacteria by negative feedback control. Iron normally enhances the synthesis of a heme compound which is the final product inhibiting ALA synthetase. This enzyme is the first enzyme in the synthesis of the intermediate (ALA) for pigment synthesis. ALA synthetase activity could be increased (2.15-fold) by adding ferric citrate (Fe^{3+}) instead of $FeSO_4$ (Fe^{2+}) (ref. 20).

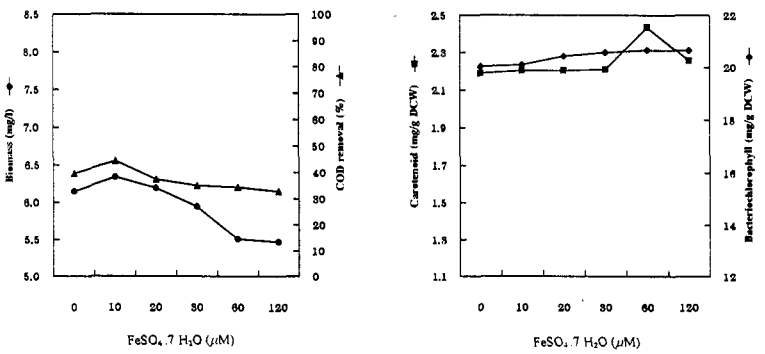

Fig.2 Effect of the concentrations of ferrous sulphate on growth, COD removal and pigment synthesis of *Rhodocyclus gelatinosus* R7 grown in tuna condensate medium supplemented with yeast extract (3 g/l) under anaerobic-light condition at room temperature.

The effect of magnesium chloride is presented in Fig. 3. Mg^{2+} showed no effect on growth in the range of 0.5-2 mM which was similar to the results for *Rhodospirillum rubrum* G-9 BM (ref. 16). The biomass increased

from 6.24 to 7.39 and 8.10 gl^{-1} as the concentration of $MgCl_2$ increased from 2 to 5 and 10 mM, respectively and decreased thereafter. The influence of magnesium may be due to the requirement of Mg in the phosphorylation reaction and as an activator for many enzymes (ref. 21). The COD removal achieved at 5 and 10 mM $MgCl_2$ after 5 days cultivation was 46% and 48%, respectively. Slightly higher pigment content was observed at 0.5 mM $MgCl_2$ with the carotenoid and bacteriochlorophyll contents of 2.33 and 20.61 mg g^{-1} DCW, respectively. This concentration (0.5 mM) was reported to be the optimal concentration for the synthesis of vitamin B_{12} (47 $\mu g\ g^{-1}$) from *Rhodospirillum rubrum* G-9BM (ref. 16). Increased concentrations of $MgCl_2$, however, markedly lowered the pigment content especially at concentrations higher than 5 mM $MgCl_2$. The concentration of 5 mM was selected for subsequent work.

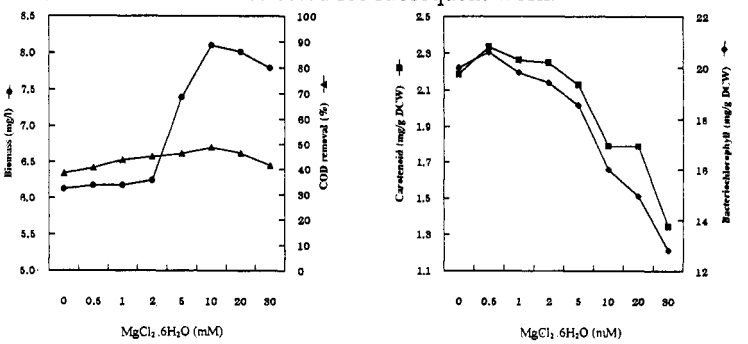

Fig.3 Effect of the concentrations of magnesium chloride on growth, COD removal and pigment synthesis of *Rhodocyclusgelatinosus* R7 grown in tuna condensate medium supplemented with yeast extract (3 g/l) under anaerobic-light condition at room temperature.

The effect of pH was studied by comparing the results obtained from control and no control of pH during the cultivation of *R. gelatinosus* R7 in tuna condensate medium supplemented with the yeast extract (3 gl^{-1}). Under controlled pH, the biomass increased 1.7-fold; from 3.69 gl^{-1} to 6.61 gl^{-1} after 4 days cultivation. The COD removals were 54% and 36% under pH control and no pH control conditions, respectively. Without pH control, the pH started to increase after 40 h and reached pH 7.7 after 4 days cultivation. This may indicate the degradation of protein in the tuna condensate and the release of ammonia and CO_2. Controlling the pH was also found to enhance the production of ALA from *Rhodobacter sphaeroides* IFO 12203 as the pH was maintained at pH 6.8-7.0 and the maximum ALA production (16 mM) was obtained at pH 7.0 (ref. 22).

Supplementation of $MgCl_2$ (5 mM) in the batch culture with controlled pH could increase the maximum biomass to 8.14 gl^{-1} after 5 days cultivation. This was higher than that (6.70 gl^{-1}) without the added $MgCl_2$. The protein content was also higher; 53% (w/w) compared to 50% (w/w) respectively. The concentrations of carotenoid and bacteriochlorophyll were 2.13 and 21.83 mg g^{-1}, respectively. The COD removal reached 51% after 3 days cultivation and increased slowly to 59% after 5 days cultivation.

The effect of repeated addition of the fresh tuna condensate into the culture broth is shown in Fig. 4. With the first addition after 40 h cultivation, the biomass reached 8.49 gl^{-1} only after 3 days (80 h) incubation. Accumulation of the biomass to 11.21 and 12.27 gl^{-1} was achieved at the end of the second and the third additions, respectively. The COD reductions were 66, 66 and 46% after 3, 5 and 7 days cultivation, respectively. The diminished COD removal correlated with a decrease in the specific growth rate after 7 days cultivation. This may have been due to either an accumulation of some metabolic products which may cause growth inhibition or repression, or to the shortage of some growth factors contained in the yeast extract. The specific growth rate of *Rhodopseudomonas capsulata* was reported to increase from 0.015 h^{-1} to 0.053 h^{-1} by adding histidine and glutamic acid (5 mgl^{-1} each) into the medium (ref.

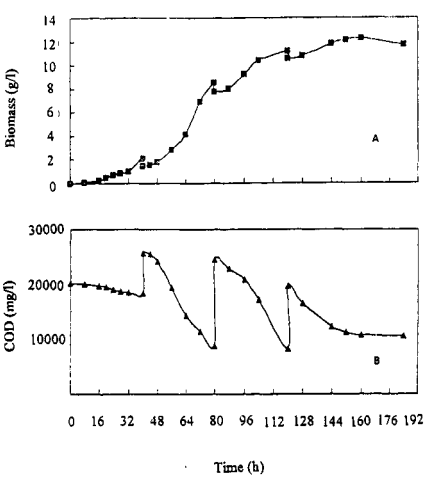

Fig.4

4). The cells of *R. gelatinosus* R7 contained 56% (w/w) protein, 2.21 mg carotenoid and 22.52 mg bacteriochlorophyll/g^{-1} dry cell weight.

CONCLUSIONS

Tuna condensate could be utilized as culture medium for *Rhodocyclus gelatinosus* R 7 grown under anaerobic-light conditons. The cell mass was increased by the addition of yeast extract (3 gl^{-1}) and 5 mM $MgCl_2$ into the tuna condensate medium as well as controlling the pH at 7.0. Cobalt chloride and ferrous sulphate were found to have no effect in the increase of biomass. The highest concentration of biomass obtained under the above conditions was 8.14 gl^{-1} with the COD removal of 59% after 5 days cultivation. The equal value for biomass concentration was obtained within 3 days after the first addition of the tuna condensate at 40 h cultivation. Further increases of biomass to 11.21 and 12.27 gl^{-1} were achieved after a second and the third addition respectively. The COD removal remained constant at 66% during the first 5 days cultivation but decreased to 46% after 7 days. The cells produced contained 56% protein, 2.21 mg carotenoid and 22.52 mg bacteriochlorophyll/g dry cell weight.

ACKNOWLEDGEMENT

The authors wish to thank the Royal Thai Government for the financial support of this project.

REFERENCES

1. Prasertsan, P. & Choorit, W. (1988). Problem and solution of the occurrence of red colour in wastewater of seafood processing plant. *Songklanakarin J. Sci. Technol.* **10**: 439-446.
2. Prasertsan, P., Choorit, W. & Suwanno, S. (1993b). Optimisation for growth of *Rhodocyclus gelatinosus* in seafood processing effluents. *World J. Microbiol. Biotechnol.* **9(5)**: 590-592.
3. Sawada, H., Parr, R.C. & Rogers, P.L. (1977). Photosynthetic bacteria in waste treatment : role of *Rhodopseudomonas capsulata* with agricultural/industrial effluents. *J. Ferment. Technol.* **55 (4)** : 326-336.
4. Sawada, H. & Rogers, P.L. (1977). Photosynthetic bacteria in waste treatment : pure culture studies with *Rhodopseudomonas capsulata. J. Ferment. Technol.* 55(4) : 297-310.
5. Sasaki, K., Noparatnaraporn, N., Hayashi, M., Nishizawa, Y. & Nagai, S. (1981). Single-cellprotein production by treatment of soybean wastes with *Rhodopseudomonas gelatinosa. J. Ferment. Technol.* **59 (6)** : 471-477.
6. Kobayashi, M. & Kurata, S. (1978). The mass culture and cell utilisation of photosynthetic bacteria. *Process Biochem.* **13(9)**: 27-30.
7. Prasertsan, P., Choorit, W. & Suwanno, S. (1993a). Isolation, identification and growth conditions of photosynthetic bacteria found in seafood processing wastewater. *World J. Microbiol. Biotechnol.* **9(5)**: 590-592.
8. Kohlmier, E.F. and Gest, H. 1951. A comparative study fermentations of organic acids by *Rhodopseudomonas sphaeroides. Biochem. J. 62 : 78-93.*
9. Hirayama, O. (1968). Lipid and lipoprotein complex in photosynthetic tissue 4 lipid and pigments of photosynthetic bacteria. *Agric. Biol. Chem.* **32** : 34-41.
10. APHA, AWWA & WPCF. (1985). Standard Methods for the Examination of Water and Wastewater. 16th ed. American Public Health Association, Washington, D.C.
11. A.O.A.C. (1984). Official Methods of Analysis of the Association of Official Chemists. 14th ed. The Association of Official Analytical Chemists. Washington, D.C.
12. Kerven, G. (1980). *Applications of atomic absorption spectroscopy to the analysis of biological materials.* Department of Agriculture. University of Queensland.
13. Bridson, E. Y. & Brecker, (1970). A: Design and formulation of microbial culture media. In: *Methods in Microbiology.* vol. 3. eds. A. J. R. Norris & D. W. Ribbons. Academic Press. London, pp.229-295.
14. Lascelles, J. (1956). The synthesis of porphyrins and bacteriochlorophyll by cell suspension of *Rhodopseudomonas sphaeroides. Biochem. J.* **62** : 78-93.
15. Morikawa, H., Hayashi, M. & Kamikubo, T. (1971). Utilisation of hydrocarbon by micro-organism (IV) : Utilisation of hydrocarbons and vitamin B_{12} production by *Rhodopseudomonas sphaeroides. J. Ferment. Technol.* **49(9)**: 803-808.
16. Hirayama, O. & Katsuta, Y. (1988). Simulation of vitamin B_{12} formation in *Rhodospirillum rubrum* G-9 BM. *Agric. Biol. Chem.* **52(11)** : 2949-2951.
17. Sasaki, K., Hayashi, M. & Nagai, S. (1978). Growth on propionate-acetate media of *Rhodopseudomonas sphaeroides* and its vitamin B_{12} biosynthesis. *J. Ferment. Technol.* **56(3)**: 200-206.
18. Cauthen, S.E., Pattison, J. R. & Lascelles, J. (1967). Vitamin B_{12} in photosynthetic bacteria and methionine synthesis by *Rhodopseudomonas spheroides. Biochem. J.* **102**: 774-781.
19. Thanarakpong, D. 1982. Cultivation of *Rhodopseudomonas* spp. for the sources of protein, pigment and vitamin B_{12}. Master of Science Thesis. Kasetsart University, Bangkok.

20. Burnham, B.F. & Lascelles, J. (1963). Control of porphyrin biosynthesis through a negative-feedback mechanism. *Biochem. J.* **87** : 462-472.
21. Shipman, R.H., Fan, L.T. & Kao, I. C. (1977). Single-cell protein production by photosynthetic bacteria. *Adv. Appl. Microbiol.* **21** : 161-181.
22. Sasaki, K., Tanaka, T., Nishio, N. & Nagai, S. (1993). Effect of culture pH on the extracellular production of 5-aminolevulinic acid by *Rhodobacter sphaeroides* from volatile fatty acids. *Biotechnol. Lett.* **15(8)** : 859-864.