CHAPTER IV

OPTIMIZATION FOR GROWTH AND TOTAL CAROTENOIDS PRODUCTION OF *RHODOBACTER* SP. TM11B FOR USING IN BLACK TIGER SHRIMP FEED

Introduction

Photosynthetic bacteria have been intensively studied for application as single cell protein (SCP) in many animal diets, with the advantages that they can converse organic waste or environmental pollutants into useful materials (Varati, 1984; Prasertsan *et al.,* 1993; Tanaka *et al.*, 1994; Yamada *et al.*, 1997; Kim *et al.*, 2000). Some evidence has been reported on the application of photosynthetic bacteria in the aquaculture industries, mostly as a larval feed for freshwater fish (Kim *et al.*, 2000). Moreover, photosynthetic bacteria can produce large amounts of many carotenoid pigments (e.g. the major group of neurosporene, spirilloxanthin, chloroxanthin, spheroidene, spheroidenone, okenone and lycopene ; the minor group of rhodopin, isorenieratene, chlorobactene, β-carotene and γ-carotene (Staley *et al*., 1989; Takaichi, 2001). By the conversion of two molecules of geranylgeranyl diphosphate (GGDP) to phytoene follow by desaturation of phytoene to neurosporene. And from the level of neurosporene, the carotenoid biosynthesis pathway of different organisms branches to generate the tremendous diversities of carotenoids found in nature (Armstrong, 1994). Pradal (1993) studied the coloration and growth of rainbow trout, using diet supplemented with synthetic astaxanthin or photosynthetic bacteria *Rhodobacter capsulatus* compared to unsupplemented diet. It was found that 0.01 % incorporation of *Rhodobacter capsulatus* was able to increase fish growth compared with those fed unsupplemented diet and diet supplemented with astaxanthin. Moreover, the coloration of the rainbow trout flesh was almost at the same level as that of fish fed diet supplemented with astaxanthin. Furthermore, the

effect of *Rhodobacter capsulatus* pigments in live feed on the lipid peroxide in the juvenile Japanese flounder was investigated (Okimasa *et al*., 1992). In the fish fed rotifers which were reared with photosynthetic bacteria, the amount of lipid peroxide in the fish on day 30 after feeding trial was lower than that in the control group. The mortalities on day 30 were also lower than those of control. Kiriratnikom *et al.* (2004) reported that the juvenile black tiger shrimp fed test diet supplemented with 5% freeze-dried *Rhodobacter* sp. TM11B had higher total carotenoids content when compared to the control without carotenoids supplementation; this bacterial strain also increased the phenoloxidase activity of the granulocytes and no evidence of histological changes were observed in the shrimp gut. The mass production for highest carotenoids content with a practical culture medium of *Rhodobacter* sp. TM11B seems to be an important point for the application of this bacterial strain as a natural source of carotenoids in shrimp diet. However, the growth and carotenoids production in the photosynthetic bacteria depend on several factors, Prasertsan *et al.* (1993b) studied the optimum levels of shrimp-bleaching water and tuna condensate as substrates for *Rhodocyclus gelatinosus.* The results showed that 1 : 10 of tuna condensate at the pH 8 under illumination intensity of 1000 Lux give the maximal growth after the incubation period of 120 hr. Moreover, the medium supplementation with magnesium chloride (5 mM) also enchanced the growth of *Rhodocyclus gelatinosus* (Prasertsan *et al*., 1997). However, the carotenoids composition of the photosynthetic bacteria can be changed by the source of substrate and metal ions in the culture medium (Britton, 1983; Takaishi, 2001). In the application of bacterial biomass in aquatic animal feed, it is necessary to minimize the production cost of the feed stuff, so, to be economically feasible, it is necessary to optimize the medium composition using a practical substrate with minimal chemical supplements for growth and total carotenoids content of *Rhodobacter* sp. TM11B.

Materials and methods

Medium

G5 medium; 3.5 g/l malic acid, 4 g/l glutamic acid, 0.12 g/l KH_2PO_4, 0.18 g/l K_2HPO_4, 5 g/l yeast extract, 5 g/l peptone (Kohlmiler and Gest, 1951) containing 3% sodium chloride were used as starter and optimization- base medium. The initial pH was adjusted to 7.0.

Microorganism

Rhodobacter sp. TM11B, the isolate from water in shrimp farm at Satun province, which previously had indicated an increase in pigmentation in black tiger shrimp (from Chapter III) was used.

Analytical methods

At 0, 24, 48, 72, 96, 120, 144 and 168 hr of incubation periods, the cells from each testing system were harvested to determined the cell mass in culture medium by the method adapted from Kim *et al.* (1999). Dry cells were obtained by centrifugation at 10,000 rpm for 10 min at 4 ^{0}C in glass centrifuge tubes, washed 3 times with 3 % NaCl solution, and dried at 105^{0}C for 24 h, weighing the dry cell mass in each tube and calculating mg dry weight / ml of culture medium.

The total carotenoids contents were determined by centrifugation at 10,000 rpm for 10 min at 4 ^{0}C to collect the cell pellet and then transferring to determine the cell pigment (total carotenoids content) following the method modified from Hirayama (1968) which were discribed in Chapter II.

Optimization studies

The inoculum of *Rhodobacter* sp. TM11B was prepared in G5 medium and transferred (10% v/v) into the

duplication of 330 ml flasks with contain 300 ml of the test medium. The culture was incubated under anaerobic-light (1,500 lux) condition, which gives the highest total carotenoids production (Chapter II). Samples were taken every 24 h, then biomass and total carotenoids were measured. The effects of nutrients supplementation were studied as follow :

1. Effect of yeast extract : peptone supplementation

Yeast extract : peptone were supplemented in basal G5 standard medium at a ratio of 0:0, 1:1, 2:2, 3:3, 4:4 and 5:5 g/l respectively.

2. Effect of carbon sources

Malic acid, glutamic acid, succinic acid, monosodium glutamate, glucose and sucrose were used as carbon sources in basal G5 medium which contained 4 g/l of glutamic acid as nitrogen source. Each carbon sources were added at a concentration of 4 g/l, compared with the control medium without carbon source.

3. Optimum level of carbon source in culture medium

Carbon source which yield the highest total carotenoids production (result from 2) was selected to investigate the effect of supplementation levels at a concentration of 0, 0.5, 1, 2, 3, 4, 5 and 6 g/l respectively.

4. Effect of metal ions supplementation

Complete medium, which gave a highest total carotenoid and biomass production (result from 3) was supplemented with the difference source of metal ions as follow :

4.1. Ferrous sulphate and magnesium sulphate : By adding each substance in the basal G5 medium at a concentration of 10-100 uM and 10-100 mM respectively.
4.2. Cobalt chloride : By adding the analytical grade of cobalt chloride in the basal G5 medium at a concentration of 5, 10, 20 and 100 uM respectively.
4.3. Ferric chloride : Analytical grade ferric chloride was added in each culture medium at a concentration of 10, 40 and 80 uM.
4.4. Ferric citrate : Ferric citrate was added in each culture medium at a concentration of 5, 10, 20, 40, 80 uM compared to the basal G5 medium without ferric citrate supplementation.

5. Effect of NaCl in culture medium

The effects of NaCl were investigated in a culture medium supplemented with the appropriate metal ion that increased the total carotenoids production (result from 4). NaCl was added in culture medium at concentrations of 0-5 %.

6. Effect of pH in culture medium

The culture medium from (5) was adjusted to pH between 5-9 for study on the effect of pH on growth and total carotenoids production of *Rhodobacter* sp. TM11B.

Carotenoid analysis

Lyophilized cell of *Rhodobacter* sp. TM11B were prepared from fresh cells cultured in the G5 medium and optimized medium in the anaerobic light condition for 72 hr. To compare the composition of medium on the production of carotenoids, the crude carotenoids were extracted from dried material by the method described by Britton *et al.* (1995), Using redistilled acetone as the first extraction solvent, and diethyl ether as secondary extraction solvent; after washing with distilled

water the crude carotenoids in the ether phase were condensed using nitrogen gas. Purification and identification of each carotenoid followed Connor (1991). Small silica column chromatography was use for the preliminary separation and then each carotenoid compound were separated by 4:1 petroleum ether : diethyl ether on silica glass plate thin layer chromatography (TLC). TLC chromatograms of carotenoid compound separated from G5 and optimized medium were compared.

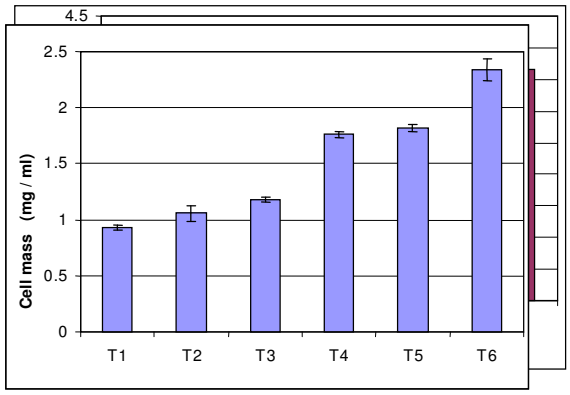
Results

Optimization studies

1. Effect of yeast extract/peptone (Y/P) supplementation

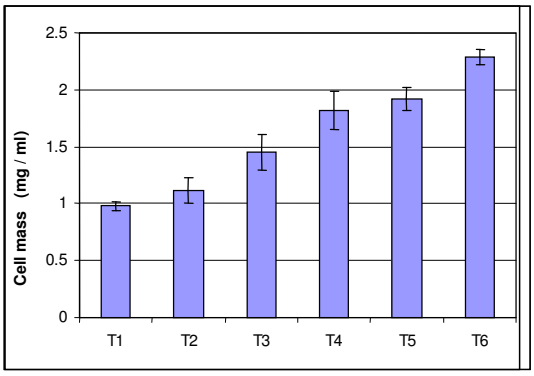
The growth and total carotenoids content in *Rhodobacter* sp. TM11B during 168 hr of cultivation are shown

in Appendix Tables 1 and 2. The highest cell mass was found in the medium containing 5 :5 yeast extract : peptone after 96 hr of culture periods (2.34±0.10 mg/ml). The total carotenoids content reached the highest concentration after 96 h of incubation period in the *Rhodobacter* sp. TM11B cultured in the medium supplemented with 3:3-5:5 yeast extract : peptone (Fig 4-1 and 4-2). After 96-120 hr of culture periods, cell mass were not significantly different between the medium containing 3:3 to 4:4 yeast extract : peptone (1.76±0.03 - 1.92±0.10 mg/ml). Total carotenoids content was highest and not significantly different among 3:3-5:5 Y/P in culture medium (3.44±0.20 - 3.85±0.07 mg/g).



(a) **(b)**

Figure 4-1 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium contain each level of Y/P after cultivation for 96 hr. (T1 = control, without yeast extract and peptone; T2 = 1:1 g/l yeast extract : peptone; T3 = 2:2 g/l yeast extract : peptone; T4 = 3:3 g/l yeast extract : peptone; T5 = 4:4 g/l yeast extract : peptone; T6 = 5:5 g/l yeast extract : peptone)

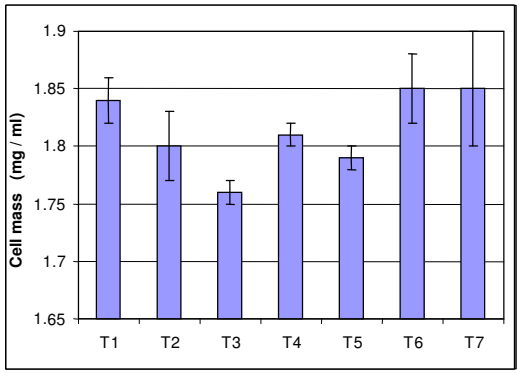


(a) **(b)**

Figure 4-2 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium contain each level of Y/P after cultivation for 120 hr. (T1 = control, without yeast extract and peptone; T2 = 1:1 g/l yeast extract : peptone; T3 = 2:2 g/l yeast extract : peptone; T4 = 3:3 g/l yeast extract : peptone; T5 = 4:4 g/l yeast extract : peptone; T6 = 5:5 g/l yeast extract : peptone)

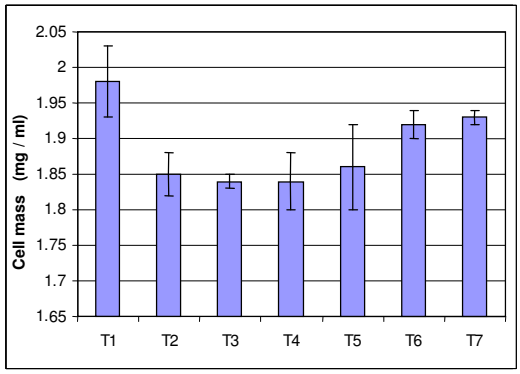
2. Effect of carbon source

The effects of each carbon source in the medium with 3:3 yeast extract : peptone given in the Appendix Tables 3 and 4. The cell mass was highest in all carbon sources after 120 hr of the incubation period (1.84$\pm$0.01 - 1.98$\pm$0.05 mg/ml) (Fig. 4-3 and 4-4). The total carotenoids content were not diferent during 96-120 hr (3.33$\pm$0.03 - 3.82$\pm$0.06 mg/g) but during 144-168 hr the total carotenoids in the *Rhodobacter* sp. TM11B was reduced in culture medium containing sucrose.



(a) **(b)**

Figure 4-3 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium contain each carbon source after cultivation for 96 hr. (T1 = control, without addition carbon source; T2 = 4 g/l malic acid; T3 = 4 g/l glutamic acid; T4 = 4 g/l monosodium glutamate; T5 = 4 g/l glucose; T6 = 4g/l sucrose; T7 = 4 g/l succinic acid)

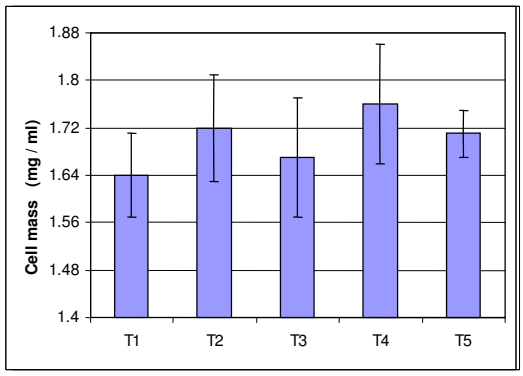


(a) **(b)**

Figure 4-4 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium contain each carbon source after cultivation for 120 hr. (T1 = control, without carbon source; T2 = 4 g/l malic acid; T3 = 4 g/l glutamic acid; T4 = 4 g/l monosodium glutamate; T5 = 4 g/l glucose; T6 = 4g/l sucrose; T7 = 4 g/l succinic acid)

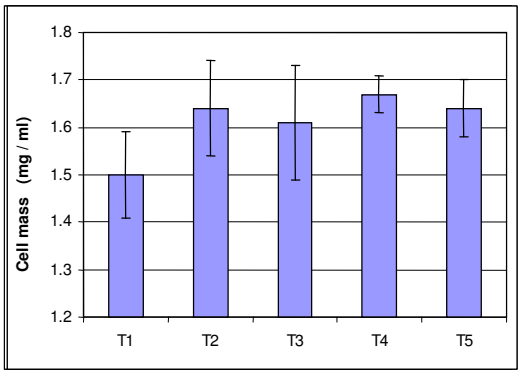
2.1 Effects of glutamic acid and monosodium glutamate as C and N source.

Because it was found that the all carbon sources give the highest total carotenoids production, especially the glutamic acid and mono sodium glutamate, which have the lowest cost when compared to other substances, the comparison of both economically beneficial carbon and nitrogen sources was also investigated. After the cultivation periods of 96 hr, the growth of *Rhodobacter* sp. TM11B was highest in all basal medium (containing 4 g/l glutamic acid) which was supplemented with either 4 or 8 g/l of glutamic acid or monosodium glutamate (1.64±0.07 - 1.76±0.10 mg/ml) (Appendix Table 5 and 6, Fig 4-5 and 4-6). There were not significantly difference (P>0.05) in the total carotenoids content among treatment after 96 hr of incubation period, but after 120 hr, the total carotenoids were lowest in the basal medium (3.06+ 0.24 mg/g). According to the results of these studies, it was noted that they was no statistical difference (P>0.05) among glutamic acid and mono sodium glutamate in means of cell mass and total carotenoids production in *Rhodobacter* sp. TM11B.



(a) **(b)**

Figure 4-5 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium containing glutamic acid and mono sodium glutamate after cultivation for 96 hr. (T1 = control, without glutamic acid and mono sodium glutamate; T2 = 4 g/l glutamic acid; T3 = 8 g/l glutamic acid; T4 = 4 g/l monosodium glutamate; T5 = 8 g/l monosodium glutamate).



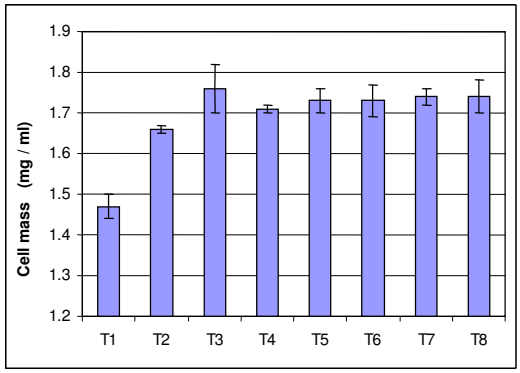
(a) **(b)**

Figure 4-6 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium containing glutamic acid and mono sodium glutamate after cultivation for 120 hr. (T1 = control, without glutamic acid and mono sodium glutamate; T2 = 4 g/l glutamic acid; T3 = 8 g/l glutamic acid; T4 = 4 g/l monosodium glutamate; T5 = 8 g/l monosodium glutamate).

3. Optimum concentration of monosodium glutamate for growth and total carotenoids production

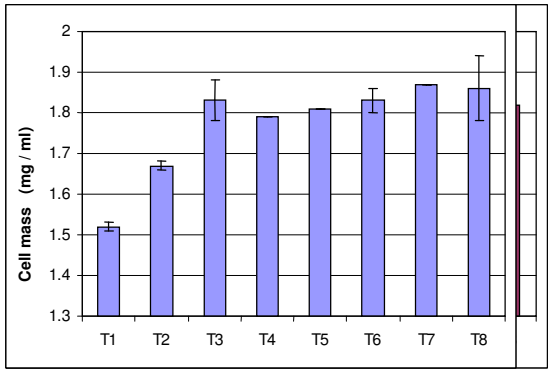
The cell mass of *Rhodobacter* sp. TM11B which were cultured in different levels of mono sodium glutamate increased with the incubation time and reached the maximum level (1.67$\pm$0.01 - 1.87$\pm$0.00 mg/ml) in 120 hr (Appendix Table 7, Fig 4-8). There were no statistical differences in cell mass in the group cultured in the medium containing 1-6 g/l of mono sodium glutamate during 96-120 hr of the incubation period. As well as

the total carotenoids production which increased with the incubation time and was not significantly different among treatments during 96-120 hr (3.58±0.10 - 4.02±0.06 mg/g) (Appendix Table 8, Fig 4-7 and 4-8). From the results, it was found that 1-6 g/l of mono sodium glutamate are the appropriate levels for cell mass production, so 1 g/l of mono sodium glutamate was chosen to be added in the medium in further studies.



(a) **(b)**

Figure 4-7 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium contain each level of mono sodium glutamate after cultivation for 96 hr. (T1 = control, without mono sodium glutamate; T2 = 0.5 g/l monosodium glutamate; T3 = 1 g/l monosodium glutamate; T4 = 2 g/l monosodium glutamate; T5 = 3 g/l monosodium glutamate; T6 = 4 g/l monosodium glutamate; T7 = 5 g/l monosodium glutamate; T8 = 6 g/l monosodium glutamate).



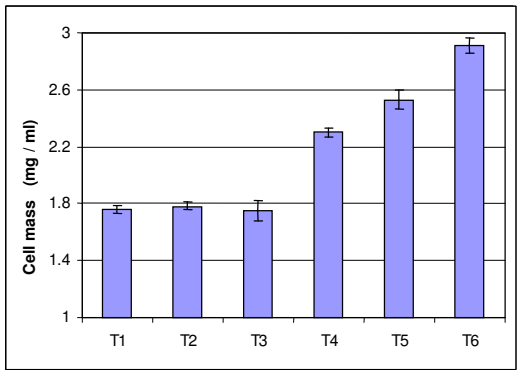
(a) **(b)**

Figure 4-8 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium containing each level of mono sodium glutamate after cultivation for 120 hr. (T1 = control, without mono sodium glutamate; T2 = 0.5 g/l monosodium glutamate; T3 = 1 g/l monosodium glutamate; T4 = 2 g/l monosodium glutamate; T5 = 3 g/l monosodium glutamate; T6 = 4 g/l monosodium glutamate; T7 = 5 g/l monosodium glutamate; T8 = 6 g/l monosodium glutamate).

4. Effects of metal ion supplements

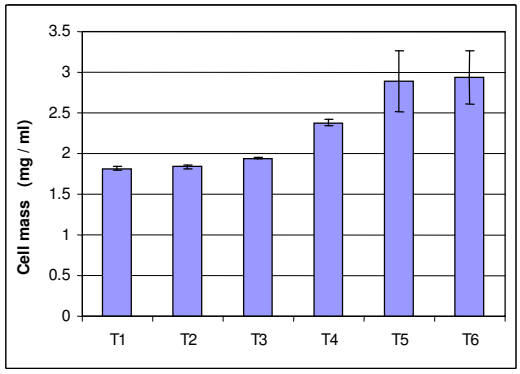
4.1 Effects of ferrous sulphate and magnesium sulphate

The effects of ferrous sulphate and magnesium sulphate were investigated in the culture medium containing 3 : 3 g/l yeast extract : peptone and 1 g/l mono sodium glutamate. Niether cell mass nor total carotenoids content of *Rhodobacter* sp. TM11B in the culture medium containing all levels of ferrous sulphate were not statistically different ($P>0.05$), but there was a positive correlation between levels of magnesium sulphate in the medium and the cell mass production (Appendix Tables 9 and 10, Fig. 4-9 and 4-10). After 120 hr of incubation periods, the highest cell mass was obtained in the *Rhodobacter* sp. TM11B cultured in the medium supplemented with 10-100 uM of magnesium sulphate (2.89 ± 0.38 - 2.94 ± 0.33 mg/ml). The total carotenoids content in *Rhodobacter* sp. TM11B was negatively correlated to the concentration of magnesium sulphate in culture medium; the lowest total carotenoids content were observed in the medium contain 10-100 uM magnesium sulphate during 96-120 hr of incubation periods (2.75 ± 0.37 - 2.98 ± 0.34 mg/g).



(a) **(b)**

Figure 4-9 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium contain ferrous sulphate and magnesium sulphate after cultivation for 96 hr. (T1 = control, without $FeSO_4$; T2 = 10 uM $FeSO_4$; T3 = 100 uM $FeSO_4$; T4 = 1 uM $MgSO_4$; T5 = 10 uM $MgSO_4$; T6 = 100 uM $MgSO_4$).



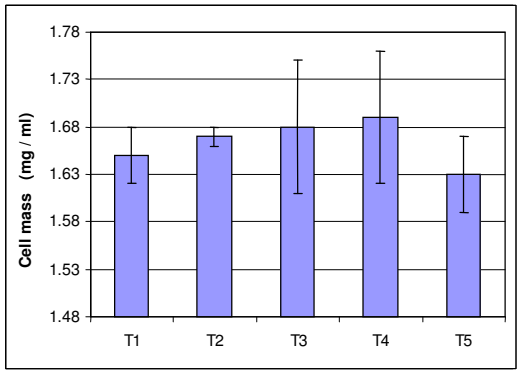
(a) **(b)**

Figure 4-10 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium contain ferrous sulphate and magnesium sulphate after cultivation for 120 hr. (T1 = control, without $FeSO_4$; T2 = 10 uM $FeSO_4$; T3 = 100 uM $FeSO_4$; T4 = 1 uM $MgSO_4$; T5 = 10 uM $MgSO_4$; T6 = 100 uM $MgSO_4$).

4.2. Effect of cobalt chloride

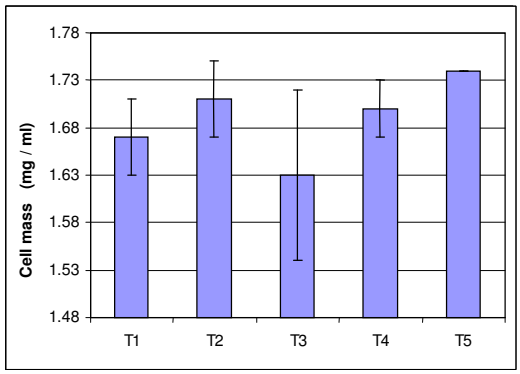
The effects of cobalt chloride on growth and carotenoids production in *Rhodobacter* sp. TM11B were

investigated in the culture medium containing 3 : 3 g/l yeast extract : peptone and 1 g/l mono sodium glutamate. After 96 hr of incubation periods, cell mass of *Rhodobacter* sp. TM11B increased with the levels of $CoCl_2$ (5-20 uM), but decreasing cell mass production was observed in the medium supplemented with 100 uM $CoCl_2$ (1.63$\pm$0.04 mg/ml). however, there were no statistical differences in the cell mass production among the treatments after 120 hr of the cultivation (Appendix Tables 11 and 12). From the analysis of total carotenoids, a negatively correlation of total carotenoids production and levels of $CoCl_2$ in culture medium was observed during 96-120 hr of incubation period (Fig. 4-11 and Fig. 4-12). The highest total carotenoids was found only in the control medium (3.89$\pm$0.01 - 4.49$\pm$0.16 mg/g).



(a) **(b)**

Figure 4-11 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium contain cobalt chloride after cultivation for 96 hr. (T1 = control, without $CoCl_2$; T2 = 5 uM $CoCl_2$; T3 = 10 uM $CoCl_2$; T4 = 20 uM $CoCl_2$; T5 = 100 uM $CoCl_2$).

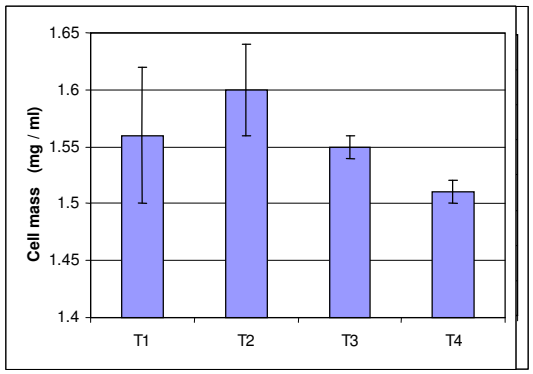


(a) **(b)**

Figure 4-12 Cell mass (mg/ml) (a) and total carotenoids (b) of *Rhodobacter* sp. TM11B in medium contain cobalt chloride after cultivation for 96 hr. (T1 = control, without $CoCl_2$; T2 = 5 uM $CoCl_2$; T3 = 10 uM $CoCl_2$; T4 = 20 uM $CoCl_2$; T5 = 100 uM $CoCl_2$).

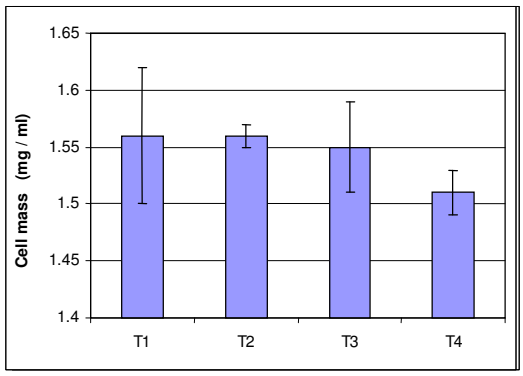
4.3 Effect of Ferric chloride

The growth and total carotenoids production of *Rhodobacter* sp. TM11B in the medium containing 0-80 uM $FeCl_2$ are shown in Appendix Tables 13 and 14. There were not significant differences ($P>0.05$) in either cell mass and total carotenoids production of *Rhodobacter* sp. TM11B in the medium contained 0-80 uM $FeSO_4$ during 96-120 hr of the cultivation (1.51 ± 0.01 - 1.60 ± 0.04 mg/ml and 3.56 ± 0.24 - 4.85 ± 0.33 mg/g) (Fig 4-13 and Fig 4-14).



(a) **(b)**

Figure 4-13 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium containing each concentration of ferric chloride after cultivation for 96 hr. (T1 = control, without $FeCl_2$; T2 = 10 uM $FeCl_2$; T3 = 40 uM $FeCl_2$; T4 = 80 uM $FeCl_2$).



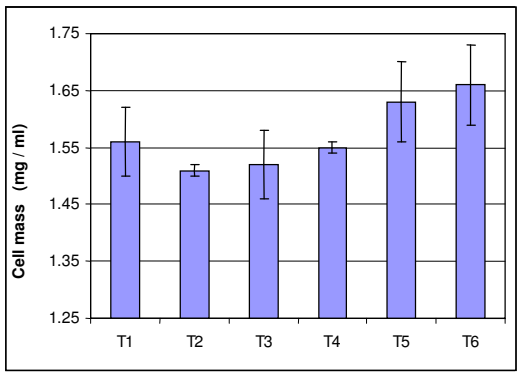
(a) **(b)**

Figure 4-14 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium containing each concentration of ferric chloride after cultivation for 120 hr. (T1 = control, without $FeCl_2$; T2

= 10 uM $FeCl_2$; T3 = 40 uM $FeCl_2$; T4 = 80 uM $FeCl_2$).

4.4 Effects of Ferric citrate

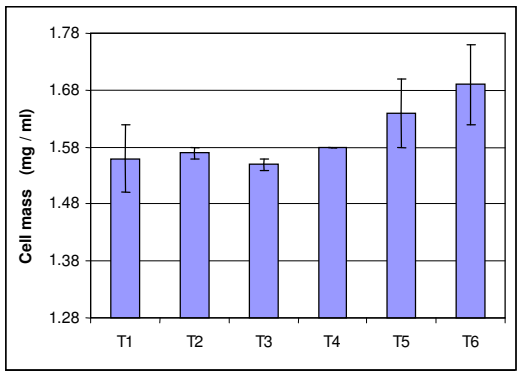
Cell mass production of *Rhodobacter* sp. TM11B was not significantly different among treatments within 96 hr of the incubation period (P>0.05). After 120 hr of cultivation, the maximal cell mass production of *Rhodobacter* sp. TM11B was found in the medium supplemented with 20-80 uM ferric citrate (1.58±0.00 - 1.69±0.07 mg/ml) (Appendix Tables 15 , Fig. 4-16). However, there was no statistical differences in cell mass production of *Rhodobacter* sp. TM11B which were cultured in the medium contained 0-40 uM ferric citrate. During 96-120 hr of cultivation, the total carotenoids contents of *Rhodobacter* sp. TM11B reach the highest level in the medium added with 5 uM ferric citrate and the lowest total carotenoids production was found with 80 uM of ferric citrate (3.36±0.23 - 4.43±0.27 mg/g) (Appendix Tables 16, Fig. 4-15 and 4-16). According to these results, the optimal level of ferric citrate for total carotenoids production in *Rhodobacter* sp. TM11B is 5 uM and will not affect the cell mass production during 96-120 hr of incubation times.



(a) **(b)**

Figure 4-15 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium contain each concentration of ferric citrate after

cultivation for 96 hr. (T1 = control, without ferric citrate; T2 = 5 uM ferric citrate; T3 = 10 uM ferric citrate; T4 = 20 uM ferric citrate; T5 = 40 uM ferric citrate; T6 = 80 uM ferric citrate).



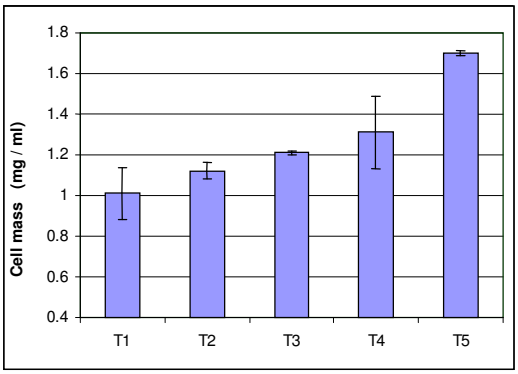
(a) **(b)**

Figure 4-16 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium contain each concentration of ferric citrate after cultivation for 120 hr. (T1 = control, without ferric citrate; T2 = 5 uM ferric citrate; T3 = 10 uM ferric citrate; T4 = 20 uM ferric citrate; T5 = 40 uM ferric citrate; T6 = 80 uM ferric citrate).

5. Effect of sodium chloride

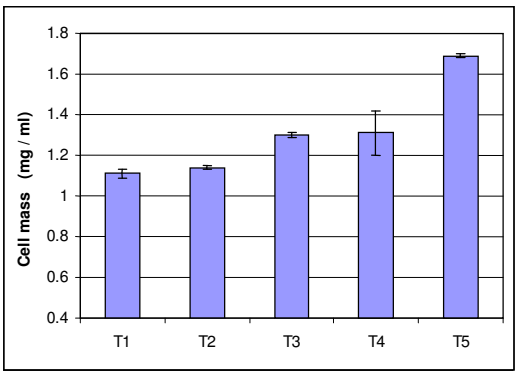
The effect of sodium chloride on growth and total carotenoids production of *Rhodobacter* sp. TM11B were investigated in the basal medium containing 3 g/l yeast extract and peptone, 1 g/l mono sodium glutamate with 5 uM ferric citrate. The maximal cell mass production of all treatments were found within 120 hr of cultivation period (Appendix Tables 17). Cell mass was highest in the medium containing 6 % NaCl

(1.70±0.01 mg/ml), however, there was no statistical difference (P>0.05) in the dry cell weight of *Rhodobacter* sp. TM11B cultured in the medium containing 4-5 % NaCl (1.21±0.01 - 1.31±0.18 mg/ml). The lowest cell mass production was observed in 2-3% NaCl (Fig 4-17 and 4-18). Even the 6% NaCl gave the highest cell mass but this level gave the lowest total carotenoids contents, folllowing the *Rhodobacter* sp. TM11B grown in 2% NaCl. The highest total carotenoids production in *Rhodobacter* sp. TM11B was found in the medium containing 5 % NaCl after incubation for 96 hr (6.69±0.26 mg/g) and was statistically different from other treatments (P<0.05). But on 120 hr of incubation these is no statistical diference in total carptenoids contents among each medium containing 3-5% NaCl.



(a) **(b)**

Figure 4-17 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium containing each concentration of sodium chloride after cultivation for 96 hr. (T1 = 2% NaCl; T2 = 3% NaCl; T3 = 4% NaCl; T4 = 5% NaCl; T5 = 6% NaCl).

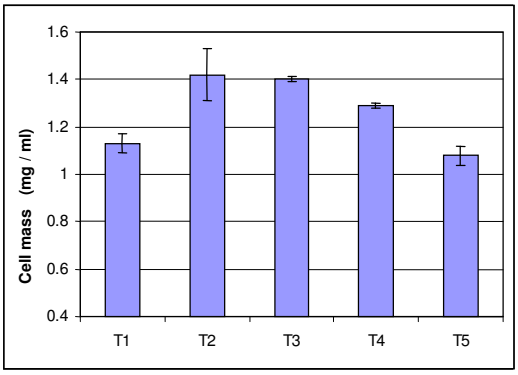


(a) **(b)**

Figure 4-18 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in medium containing each concentration of sodium chloride after cultivation for 120 hr. (T1 = 2% NaCl; T2 = 3% NaCl; T3 = 4% NaCl; T4 = 5% NaCl; T5 = 6% NaCl).

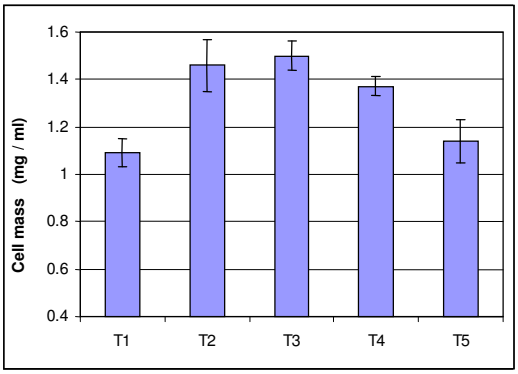
6. Effects of pH

The growth of *Rhodobacter* sp. TM11B increased with the time of cultivation but the highest cell mass production was observed in the culture medium with the pH 6-7. From Appendix Table 19, Fig 4-19, it was found that the maximal cell mass production was observed at pH 6-8 after incubation for 120 hr (1.37±0.04 - 1.50±0.06 mg/ml). As well as the total carotenoids content which are highest in the medium adjusted to pH 7 at 96 hr of the incubation periods (5.93±0.05 mg/g). These was no significant difference in the total carotenoids content in the pH 6 and 8 and the lowest carotenoids production were observed in pH 5 and 9 (Fig 4-19 and 4-20).



(a) **(b)**

Figure 4-19 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in the difference pH of culture medium after cultivation for 96 hr.
(T1 = pH 5; T2 = pH 6; T3 = pH 7; T4 = pH 8; T5 = pH 9)



(a) **(b)**

Figure 4-20 Cell mass (mg/ml) (a) and total carotenoids (mg/g) (b) of *Rhodobacter* sp. TM11B in the difference pH of culture medium after cultivation for 120 hr. (T1 = pH 5; T2 = pH 6; T3 = pH 7; T4 = pH 8; T5 = pH 9)

Carotenoids analysis

The thin layer chromatogram of carotenoid pigment from *Rhodobacter* sp. TM11B in the G5 or optimized medium are given in figure 4-21. There was no difference in the carotenoid composition of this bacterial strain when cultured in G5 or optimized medium used in this study.

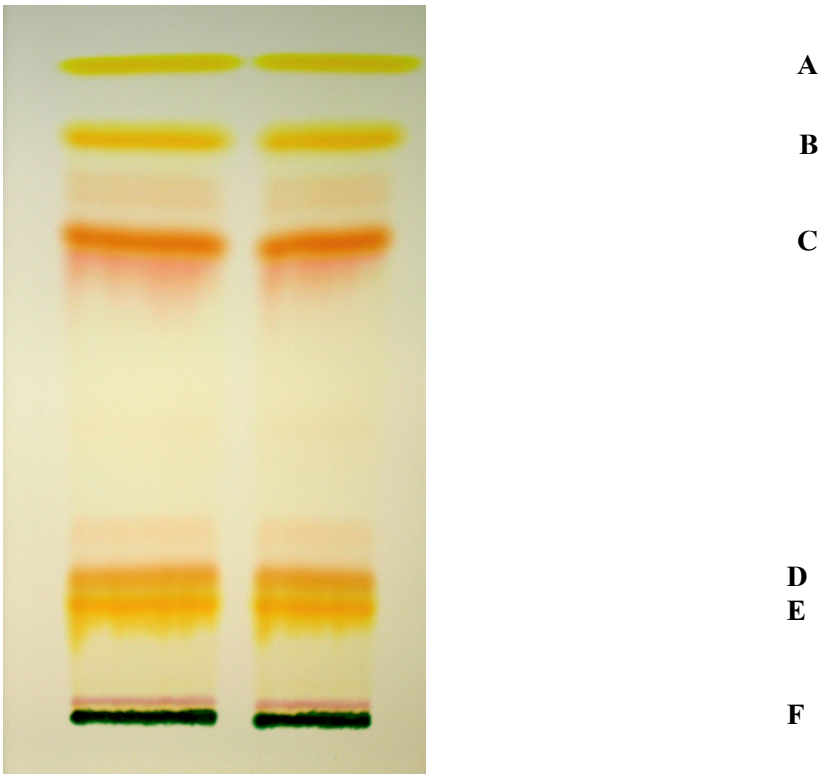


Figure 4-21 Thin layer chromatogram of carotenoids pigment from *Rhodobacter* sp. TM11B in the G5 (right) or optimized medium used in this study (left). (A= neurosporene, B = spheroidene, C= spheroidenone, D = demethylspheroidene, E = hydroxyspheroidene, F = bacteriochlorophyll a)

Discussion

The addition of yeast extract and peptone to culture medium increased the growth of *Rhodobacter* sp. TM11B, but reduced the total carotenoids content at the high levels of yeast extract and peptone. The optimum concentration of yeast extract and peptone for the highest total carotenoids content in *Rhodobacter* sp. TM11B is 3 g/l, which is similar to the result from the studies in *Rhodocyclus gelatinosus* R7 by Prasertsan *et al.* (1997) but such level of yeast extract is the highest concentration in study. As well as in the report from Sawada and Roger (1977), the highest biomass was obtained at the highest concentration of yeast extract in culture medium. Yeast extract and peptone seem to affect the growth and carotenoids production of *Rhodobacter* sp. TM11B by the presence of nitrogen, carbon and numbers of growth factors including biotin and thiamine. However, at the higher concentration, it seems to inhibit the carotenoids synthesis. Biotin is needed for the formation of 5-aminolevulinic acid (ALA) which is an intermediate of bacteriochlorophyll synthesis (Presertsan *et al.* 1997).

In general, bacteriochlorophyll a and b were produced in the photosynthetic bacteria from geranylgeranyl pyrophosphate (GGPP), which is the same intermediate for the synthesis of prephytoene pyrophosphate (PPPP) for the formation of the first carotenoid, phytoene (Britton, 1983; Armstrong, 1994), so the increase in the synthesis of bacteriochlorophyll a from GGPP may tend to decrease the substrate available for the formation of PPPP. And this phenomenon will probably lead to a decrease in the production of phytoene from PPPP. However, the addition (4 g/l) of malic acid, glutamic acid, succinic acid and glucose in the culture medium contain 4 g/l of glutamic acid did not affect the growth and carotenoids production of the photosynthetic bacteria

Rhodobacter sp. TM11B. Glutamic acid was found to enhance the assimilation of propionate by *Rhodopseudomonas capsulata* (Morikawa *et al.*, 1971) and increase the biomass production, from our studies, the glutamic acid and monosodium glutamate at the same levels also did not affect the growth and total carotenoids production of *Rhodobacter* sp. TM11B. For the economical reasons, the food grade monosodium glutamate (99.9 %) is available with the lowest cost, so it is a practical strategy to apply this substrate for the production of *Rhodobacter* sp. TM11B as carotenoids source in the large scale. From the study on the optimum level of monosodium glutamate for growth and carotenoids production of *Rhodobacter* sp. TM11B, it was found that the growth and total carotenoids production were not different at the levels of 1-5 g/l monosodium glutamate but the highest cell mass was obtained in the medium containing 6 g/l monosodium glutamate with the lowest carotenoids content. The ratio of carotenoids produced in *Rhodobacter* sp. TM11B to the amount of monosodium glutamate in culture medium at the highest point (120 hr of the incubation periods) are 13.16 follow by 7.08 ug/g monosodium glutamate in the group cultivated in 0.5-1 g/l monosodium glutamate respectively compared to 3.50-1.25 in the 2-6 g/l monosodium glutamate. Even the culture medium containing 0.5 g/l monosodium glutamate seems to give the highest carotenoids level but the growth of *Rhodobacter* sp. TM11B at this concentration is very low when compare that with 1 g/l monosodium glutamate, so the suitable level of monosodium glutamate for the large scale production of *Rhodobacter* sp. TM11B is 1 g/l. From the studies on the effects of the concentrations of metal ions on the growth and total carotenoids production of *Rhodobacter* sp. TM11B it was found that the ferrous ion did not affect the growth and total carotenoids content in *Rhodobacter* sp. TM11B cultured in all levels of $FeCl_2$.

From the studies on the effect of $FeSO_4$ in *Rhodocyclus gelatinosus* the addition of 10 uM $FeSO_4$ increased the cell mass of this bacteria but did not increase in the

production of carotenoids and bacteriochlorophyll (Prasertsan *et al.*, 1997). The growth of *Rhodobacter* sp. TM11B was increased in the present of magnesium sulphate. Magnesium is required for phosphorylation reactions and as an activator for many enzymes (Shipman *et al.*, 1977). The result of magnesium sulphate on the growth of *Rhodobacter* sp. TM11B from our studies is in agreement with the use of magnesium chloride in *Rhodocyclus gelatinosus* R7 and the addition of magnesium chloride for *Rhodospirillum rubrum* G-9BM (Hirayama and Katsuta, 1988; Prasertsan *et al.*, 1997). However, the increment of magnesium ions lead to a decrease in the production of total carotenoids, 5-30 mM of MgCl increased the cell mass in *Rhodocyclus gelatinosus* R7 but the pigment contents were decreased around 40 % from the control (Prasertsan *et al.*, 1997). The reduction of total carotenoids content at high concentration of magnesium ion was also found in the *Rhodobacter* sp. TM11B; the total carotenoid in the cell cultured in 10-100 mM $MgSO_4$ were 22 % lower than the control without the addition of magnesium ion. Magnesium is needed in the metal chelation for the formation of bacteriochlorophyll a by the introduction of the magnesium ion into the molecule of protoporphyrin IX to the protochlorophyllide a and chlorophyllide a respectively (Britton, 1983). Chlorophyllide a will be esterified with phytol, farnesol or geranylgeraniol to complete bacteriochlrophyll a. At this step photosynthetic bacteria need farnesylpyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) as the sources of esterified phytol (Britton, 1983). But both FPP and GGPP are importance intermediates in the biosynthesis of carotenoid (phytoene) so if the FPP and GGPP were mostly used in the formation of bacteriochlorophyll in the present of high magnesium ion, the biosynthesis of carotenoids will be decreased by the lack of intermediate substances.

The growth of *Rhodobacter* sp. TM11B was constant at all levels of cobalt chloride, but the total carotenoids content decreased with increasing cobalt ion. The highest carotenoids production found in *Rhodobacter* sp. TM11B cultured with the

medium containing 5 uM ferric citrate, the cell mass were not difference in all levels of ferric ion. This result is in agreement with the report in *Rhodopseudomonas spheroides* S which required the 4 mg/l ferric citrate for growth and synthesis of pigments (Thanarakpong, 1982). However, the total carotenoids of *Rhodobacter* sp. TM11B decreased in the high concentration of ferric citrate (10-100 uM), high concentration of iron inhibits growth and pigment synthesis of photosynthetic bacteria by negative feedback control because iron will enhance the synthesis of heme which is the last product in the porphyrin synthesis by aminolevulinic acid synthetase, so finally high concentration of heme inhibited the activity of enzyme in the pigment synthesis (Prasertsan *et al.*, 1997). The optimum pH for the growth and carotenoids production of *Rhodobacter* sp. TM11B (pH 7.0) is correlated to the *Rhodobacter spheroides* IFO 12203 (Sasaki *et al.*, 1993) and *Rhodocyclus gelatinosus* R7 (Presertsan *et al.*, 1993a).

The *Rhodobacter* sp. TM11B seems to be a halophilic photosynthetic bacteria; the growth of this bacteria increased with increasing sodium chloride, however at 6 % NaCl a decrease of total carotenoids content was observed. Our studies did not determine the effect of light intensity for the growth of *Rhodobacter* sp. TM11B, as the report in *Rhodocyclus gelatinosus* R7 that lower light intensity increased the pigment synthesis (Presertsan *et al.*, 1993a), the light intensity from the tungsten lamp in our studies was 1,500 Lux, which is close to the optimum intensity for pigment synthesis in *Rhodocyclus gelatinosus* R7. The result from this study shown that the optimization medium enchance the total carotenoids content in *Rhodobacter* sp. TM11B from 2.61 $\pm$ 0.19 mg / g in G5 medium at 96 hrs of incubation periods to 5.93 $\pm$ 0.05 mg / g in modified optimized medium within 96 hrs of the incubation periods which is 227.2 % higher than normal G5 medium at the same incubation time.

References

Armstrong, G. A. 1994. Eubacteria show their true colors : Genetics of carotenoid pigment biosynthesis from microbes to plants. Journal of Bacteriology. 176 (16) : 4795-4802.

Britton, G. 1983. The biochemistry of natural pigments. Cambridge university press. Cambridge. 366 p.

Hirayama, O. 1968. Lipids and lipoprotein complex in photosynthetic tissue: 4 lipid and pigments of photosynthetic bacteria. Agricultural and Biochemical Chemistry 32 : 34-41.

Hirayama, O. and Katsuta, Y. 1988. Stimulation of vitamin B_{12} formation in *Rhodospirillum rubrum* G-9 BM. Agricultural Biology and Chemistry 52(11) : 2949-2951.

Kim, J. K. and Lee, B. K. 2000. Mass production of *Rhodopseudomonas palustris* as diet for aquaculture. Aquaculture Engineering 23 : 281-293.

Kim, J. K., Lee, B. K., Kim, S. H. and Moon, J. H. 1999. Characterization of denitrifying photosynthetic bacteria isolated from photosynthetic sludge. Aquaculture Engineering 19 : 179-193.

Kiriratnikom, S., Supamattaya, K., Prasertsan, P. and Britton, G. 2004. Effects of astaxanthin and photosynthetic bacteria on growth performance, pigmentation and immune response in black tiger shrimp (*Penaeus monodon*). 11th International Symposium on Nutrition and Feeding in Fish : Nutrition of Fish & Shellfish : Seafood for Better Human Health & Well Being. 2-7 May 2004, Phuket Arcadia Hotel, Phuket, Thailand. pp. 233.

Kohlmiler, E. F. and Gest, H. 1951. A comparative study fermentations of organic acids by *Rhodospirillum rubrum*. Journal of Bacteriology 61 : 269-282.

Morikawa, H., Hayashi, M. and Kamikubo, T. 1971. Utilization of hydrocarbon by micro-organism (IV) : Utilization of

hydrocarbon and vitamin B_{12} production by *Rhodopseudomonas sphaeroides*. Journal of Fermentation Technology 49(9) : 803-808.

Okimasa, E., Matsumoto, M., Yoshida, Y. and Amemura, A. 1992. The effect of pigments of *Rhodobacter capsulatus* on free redicals and application of the bacterium as feed to fish larvae. Nippon Suisan Gakkaishi 58 (8) : 1487-1491.

Pradal, M. 1993. Trout flesh colouring : A new photosynthetic bacteria. Aqua Revenue 47 : 29-33.

Prasertsan, P., Choorit, W. and Suwano, S. 1993a. Isolation, identification and growth conditions of photosynthetic bacteria found in seafood processing wastewater. World Journal of Microbiology and Biotechnology. 9 : 590-592.

Prasertsan, P., Choorit, W. and Suwanno, S. 1993b. Optimization for growth of *Rhodocyclus gelatinosus* in seafood processing effluents. World Journal of Microbiology and Biotechnology 9 : 593-596.

Prasertsan, P., Jaturapornpipat, M. and Siripatana, C. 1997. Utilization and treatment of tuna condensate by photosynthetic bacteria. Pure and Applied Chemistry 69 (11) : 2439-2445.

Sasaki, K., Tanaka, T., Nishio, N. and Nagai, S. 1993. Effect of culture pH on the extracellular production of 5-aminolevulinic acid by Rhodobacter spheroids from volatile fatty acids. Biotechnology Letters 15(8) : 859-864.

Sawada, H. and Rogers, P. L. 1977. Photosynthetic bacteria in waste water treatment : pure culture studies with *Rhodopseudomonas capsulata* with agricultural/industrial effluents. Journal of Fermentation Technology 55(4) : 297-310.

Shipman, R. H., Fan, L. T. and Kao, I. C. 1977. Single-cell protein production by photosynthetic bacteria. Advanced in Applied Microbiology 21 : 161-181.

Staley, J. T. Bryant, M. P., Pfening, N. and Holt, J.G. 1989. Bergey' s manual of systematic bacteriology, 3th ed. 2298 p. Baltimore : William and Wilkins.

Takaichi, S. 2001. Carotenoids and carotenogenesis in anoxygenic photosynthetic bacteria. In Frank, H. A., Young, A. J., Britton, G. and Cogdell, R. J. (eds) The Photochemistry of Carotenoids. pp. 40-69. Kluwer Acadmic Publishers Dordrecht.

Tanaka, T., Sasaki, K., Noparatnaraporn, N. and Nishio, N. 1994. Utilization of volatile fatty acids from the anaerobic digestion liquor of sewage sludge for 5-aminolevulinic acid production by photosynthetic bacteria. World Journal of Microbiology and Biotechnology. 10 : 677-680.

Tanarakpong, D. 1982. Cultivation of *Rhodopseudomonas* spp. For the sources of protein, pigment and vitamin B_{12}. Master of Science Thesis. Kasetsart University, Bangkok.

Vrati, S. 1984. Single cell protein production by photosynthetic bacteria grown on the clarified effuents of biogas plant. Applied Microbiology and Biotechnology 19 : 199-202.

Yamada, A., Miyashita, M., Inoue, K. and Matsunaga, T. 1997. Extracellular reduction of selenite by a novel marine photosynthetic bacterium. Applied Microbiology and Biotechnology 48 : 367-372.