CHAPTER IV

DISCUSSIONS

Field Survey and Isolation of Dunaliella spp. From Salt Ponds.

It was found that salt pond in Samut Songkhram with the salinity exceeding 100 ppt would be predominated with D. salina. The highest number of D. salina was found at 300 ppt salinity. Nevertheless, D. salina was not found in water samples from Chonburi because of the low salinity. Some flagellates and some dinoflagellates were found in Chanthaburi low salinity sample but nothing was found in high salinity water samples. With this regard, more exploration and isolation of new D. salina strains from Chanthaburi and others remote areas such as Pattani Province (Southern part) or salt lakes in the North-East part of Thailand should be done in the future.

D. viridis was found to be associated with D. salina in water salinity up to 250 ppt. The occurrence of D. viridis together with D. salina was commonly found in high saline lake around the world (Borowitzka, 1981). However, D. salina appears to have a higher optimum salinity than D. viridis. With this reason, D. viridis tends to remain at the bottom of a pond at 250 ppt, whereas D. salina remains active in water column at salinity up to 310 ppt (Moulton et al., 1987a).

The algal isolation by micro pipette single cell isolation was satisfactory for D. salina because the cell could be observed under an inverted microscope. However, the other methods such as streak plating and isolation on agar could be used for D. salina isolation (Haydae Montoya, 1991 (personal communication)) but this method consumed more time to ensured clone than the single cell isolation. To isolated D. salina from high saline water samples, lower salinity culture medium was recommended. The isolated cell which cultured in lower salinity (105 ppt from initial salinity of 255 ppt) could survived and divided faster than in higher salinity culture medium (Table 2).

The study on nutrient concentrations in water samples indicated that nitrate and phosphate increased with increasing salinity (Figure 11, 12). A similar result was reported by Senming et al. (1990). They found that nutrient values in saltfarm in Dongyuan, China increased with salinity while Zhang et al. (1990) reported that nutrient values in Tanggu solar saltworks in China increased with increasing salinity until the salinity reached 200 ppt which the nutrient concentration was lowered because of algal biomass in the pond.

As regards the taxonomical study on genus Dunaliella, the classification characteristics of this genus depended on cell colour, size and halotolerant capability (Lerche, 1937; Butcher, 1959). Loeblich (1982) and Al-Hasan et al. (1987) suggested that carotenoid content and carotene/chlorophyll ratio is the important character for D. salina. The isolated clones of D. salina in this experiment were

in accordance with all taxonomic description. Therefore, it could be confirmed the species name was salina.

Selection of D. salina Clone Yielding High Carotenoid Content.

Fulks and Main (1991) explained that there could be significant difference in growth among algal strains (within species) or even clones (genetically identical descendants of one asexually reproducing organism). For example, Cifuentes et al. (1992) isolated eight strains of D. salina from salt ponds in Chile. They found the different growth capacity and carotenogenesis among the isolated strains. Similarly, specific growth rates and carotenoid contents of this experiment were different among the isolated clones of D. salina. Furthermore, Borowitzka and Borowitzka (1988a) proposed the problem during to D. salina mass culture might be solved by proper strain selection.

D. salina grew well in the low salinity culture medium rather than high salinity while carotenoid content in high salinity was higher (Figure 13-18). The carotenoid content was much higher in medium which contained 20% and 30% NaCl concentration. It was clear that salinity is the important parameter for β-carotene accumuulation in D. salina (Semenenko and Abdullaev, 1980; Loeblich, 1982; Al-Hasan et al., 1987; Borowitzka et al., 1990). However, Ben-Amotz and Avron (1983) suggested that major inducing factor of β-carotene accumulation in D. bardawil was light intensity and they showed a positive interaction between salinity and light intensity on β-carotene

accumulation. Hence, D. salina needed both high light and high salt for the maximum β-carotene production. Salinity alone might not be sufficient to promote β-carotene accumulation capability of each clone. With this regard, three salinity levels were used for clone selection in this experiment. The result showed that many clones of D. salina which isolated from salt pond showed high capability on β-carotene accumulation in the high salinity. The D. salina clone number DS91008 showed the highest β-carotene content in all salinity culture medium with the maximum carotenoid content of 80.4 pg/cell in 30% NaCl (Figure 18). Thus, this strain was selected for the further experiment.

Effects of Light Intensity, Nutrient Concentration and Initial pH on Growth and Carotenoid Content of D. salina.

a) Effect of Light Intensity.

D. salina clone number DS91008 could accumulate more carotenoid in higher light intensity than the lower light while the specific growth rates were not significant different. The similar result was reported by Ben-Amotz and Avron (1983). Junmin (1990) reported that red cell of D. salina could not be produced in his experiment using different salinity. The result of this experiment can explained that light intensity (his experiment was only 6,600 lux) was insufficient. D. salina could not turned red if light intensity was lower than 10,000 lux (Figure 20).

Absorption spectra of D. salina (Figure 21) showed different spectra pattern in carotenoid (452 nm) and chlorophyll peak (647, 664 nm) in each light intensity. The amount of β-carotene increased with increasing light intensity while chlorophyll decreased. Thereafter, the result was re-confirmed by HPLC analysis of β-carotene (Figure 22, 23 and 24). It was found that β-carotene was a major pigment of the orange stage of D. salina. A detailed study of HPLC analysis on acetone extract Dunaliella was done by Schierle et al. (1992). α-Carotene and three isomers of β-carotene (All-trans, 9-cis and 13-cis) was detected with small peaks of Lutein and Zeaxanthin. In addition, Borowitzka et al. (1990) reported that proportion of the individual carotenoid in D. salina varied up to almost 100% β-carotene of total carotenoid when the algae was cultivated in 30% NaCl while Zeaxanthin levels decreased to zero. The result from this experiment showed that more than 90% of carotenoid was β-carotene. Thus, the further experiment might use only carotenoid determination to indicate the approximate level of β-carotene of the orange stage D. salina.

The discriminant analysis showed that D. salina which had low carotenoid content (green stage) had smaller size in comparison with the orange stage (Figure 25). Al-hasan et al. (1987) reported that there were two mean cell dimensions, 9x5 μm in 2.5% NaCl or 19x11μm in 30% NaCl depended on β-carotene accumulation, in their experiment. Cell size of the orange stage D. salina could reached up to 25x12-16μm in outdoor cultivation (Borowitzka and Borowitzka, 1990). Because of size variation, the taxonomical characteristics (see literatures review) would difficult to separate D. salina from others species if algal cell

cultivated in low salinity and low light intensity (green stage). Therefore, it could not be disregarded that β-carotene accumulation capability is the most important characteristic of D. salina.

b) Effect of Nitrate Concentrations.

Nitrate starvation affected both growth rate and carotenoid content of D. salina. Previously published data on D. salina (also D. bardawil) suggested that the nitrate deficiency was an inducing factor for β-carotene accumulation in addition of light intensity and salinity (Semenenko and Abdullaev, 1980; Ben-Amotz and Avron, 1983; Borowitzka and Borowitzka, 1988a; Junmin, 1990). In this experiment, carotenoid content was increased with decreasing nitrate concentration (Figure 27). The maximum carotenoid content was at 10% KNO_3 of J/1 medium. However, carotenoid content decreased when nitrate concentration declined to 1% nitrate. The similar result was reported by Ben-Amotz and Avron (1983). They studied the effect of nitrate concentrations on β-carotene content of D. bardawil. β-carotene content rose up while nitrate concentration decreased. Until nitrate concentration was near zero, β-carotene content was dropped. Moreover, the effect of nitrate deficiency on increasing carotenoid content was found not only in D. salina but also in other algae such as chlorella (Thongprasong, 1989).

In the logarithmic growth phase, carotenoid content of D. salina cultivated in 10% nitrate was significantly highest, while at the stationary phase it declined to the lowest. It is thus probable that nitrate was utilized by algal cell. Nitrate

concentration in stationary phase culture medium was less than the former medium, thus, amount of nitrate in stationary growth phase was not enough for metabolism and carotenogenesis.

c) Effect of Phosphate concentrations.

Phosphate is the major nutrient which is required for metabolic process especially on cellular respiration. Variation of phosphate concentration in algal medium could affect growth and algal metabolic products. In the current experiment, phosphate starvation could significantly reduce the growth rate of D. salina (Figure 30). However, the effect of phosphate starvation on carotenoid content was less when compare with nitrate (Figure 31). When phosphate concentration was reduced, carotenoid content would slightly increase. On the other hand, the excess amount of phosphate caused the reduction in both growth rate and carotenoid content. Nevertheless, the statistical multiple range test indicated that there was no significant difference in carotenoid content among phosphate concentrations in both log and stationary growth phase.

Junmin (1990) reported that carotenoid content of D. salina was slightly increased with the reducing phosphorus concentration. Thongprasong (1989) studied effect of phosphate concentration on carotenoid content of Chlorella. She found that growth rate and carotenoid content of Chlorella would be decreased if there was an excess phosphate concentration in culture medium. Similarly, Borowitzka and Borowitzka (1988a) reported that the optimum phosphate concentration for growth of

D. salina is about 0.02-0.025 g/l and the excess amount of K_2HPO_4 (>5g/l) would inhibit growth; the effect on carotenoid content was not reported.

d) Effect of Initial pH.

The experiment was done in order to control constant pH value of J/1 culture medium. It was found that pH could not be adjusted due to the pH buffering capacity of the medium itself. Therefore, tris-buffer was necessary added to control pH value.

It was previously reported that Dunaliella has a wide optimum pH between 7 and 9 (Ben-Amotz and Avron, 1989a) and the optimal pH for β-carotene production was 9 (Anon, 1983 cited by Borowitzka and Borowitzka, 1988a). The pH value affected not only algal cell but also liquid chemistry. The co-precipitation of carbonates, phosphate and sulphate with Ca^{2+} was found in high salinity water (more than 1 mM of Ca^{2+}) which pH more than 8 (Ben-Amotz and Avron, 1989a). Our results showed that specific growth rate of D. salina was affected due to improper pH (Figure 34). The pH 7.36 provided the maximum growth rate. The growth rate would drop if D. salina culture grew at pH lower or higher than 7.36. The maximum carotenoid content in this experiment was at pH 8.3 in stationary growth phase (Figure 36). However, in comparison with the effect of nitrate deficiency, pH has little impact on carotenoid content. The multiple range test showed that there was no significant different in both carotenoid and chlorophyll-a in log phase but the carotenoid and chlorophyll-a content was significant highest at pH 8.3 in stationary phase.

Moreover, the carotenoid to chlorophyll-a ratio was significantly highest at pH 8.7. The previous study, Junmin (1990) suggested that pH 6-8.5 did not affected growth and carotenoid content of D. salina in his experiment. Nevertheless, he found that at salinity 20°Be' (about 195 ppt) the maximum carotenoid accumulation ratio was at pH 8.0.

Mass Cultivation of D. salina in Outdoor Raceway Pond.

The isolated clone of D. salina (DS91008) was cultivated in a pilot scale 9.1 m^2 outdoor pond. During the logarithmic growth phase, an approximate doubling time of 4.6 day was obtained from the experimental pond (Figure 42). Unfortunately, because of rainy season, rain was a serious problem for the outdoor culture. Light intensity was sometime limited by the cloudy sky. Medium temperature was fluctuated to 40.7°C at day and 29.2°C at night. However, the transparency plastic sheet could protect the pond from rain. The result indicated that D. salina could be cultivated even in rainy season.

In general, the climate of Thailand is suitable for D. salina culture especially in some coastal area along the Gulf of Thailand which are used for salt evaporation pond and the semi-arid area in the north-east part of Thailand. The underground rock salt in the north-east can be a good source of high salinity water. The average temperature is rather warm, medium temperature at night is usually not lower than 25°C whereas Borowitzka and Borowitzka (1988b) suggested that low night temperature could decreased growth rate of D. salina. The rainy

season in Thailand has a duration for about 6 month from May to October but heavy rain is found in August and September. The 39 year period statistic data showed that amount of rain in rainy season was 932.1 mm in the middle part and 1104.8 mm in the north-east part, however, 70-130 mm rain was measured throughout cold and summer season (Wongwitawas, 1989). Therefore, the intensive cultivation system may be started from laboratory to starter pond in the protected pond during rain season then follow with Dunaliella mass production in January until June. On the other hand, extensive culture may be done together within the salt evaporation field by using natural strain.

In the outdoor environment, light intensity is necessary for algal photosynthesis but the excess light may inhibit growth of the algae in the pond (photoinhibition). Vonshak (1987) suggested that in most algal species, photoinhibition was observed about 60-70% of full sunlight. The environmental data during mass culture indicated that D. salina could grown well in very high light intensity. Dissolved oxygen from photosynthesis was increased with increasing light intensity without any drop (Figure 44). Although the oxygen meter which used in this experiment (YSI model 57) has the maximum measurements of oxygen only 20 ppm while oxygen in algal pond was higher, however, the correlation between light intensity and dissolved oxygen was parallel increasing. It is presumably that photoinhibition was not found in D. salina outdoor culture. Similarly, Gómez-Pinchetti et al. (1992) reported that the red D. salina could resist the high irradiance up to 2000 $\mu E/m^2/s$ without photoinhibition.

Borowitzka and Borowitzka (1990); Liangchen (1990) described a two-step culturing system, first to optimize growth condition and followed by optimizing the β-carotene accumulation. From the result, it was thus probable that low salinity and high nitrate concentration culture medium might be used to optimize growth at the beginning. After retaining maximum growth, the optimize condition for β-carotene production could proceed with increasing salinity and at reduced nitrate concentration. However, two-steps culturing system might induce the protozoa and D. viridis contamination because of the low salinity and, moreover, high cost of pond operation.

The intensive paddle-wheel raceway ponds, for example, in Israel, U.S. (Vonshak, 1990) or China (Liangchen, 1990) may not a suitable system for D. salina culture in Thailand although this system can provide high yield. The natural pond such as in Australia has much lower productivity but also lower in operation cost (Moulton et al., 1987b; Borowitzka and Borowitzka, 1990). Therefore, the study on the proper system for D. salina culture in Thailand must verified by experimentation in cooperation with the economic analysis.

During mass culture in the current experiment, when salinity decreased, protozoa and other contaminants could affect in the algal pond. Although, there was no reported on D. viridis contamination in the intensive Dunaliella pond in Israel (Ben-Amotz, personal communication). In this experiment, it was found that the protozoa which identified as flagellate phase of amoeba Heteramoeba sp. and D. viridis could contaminate in the experiment pond if salinity decrease below 190 ppt.

As reported previously from field observation, Post et al. (1983) observed 14 ciliates, 10 zooflagellates and 4 sarcodines in Hutt lagoon (over 15% NaCl) in Australia which the crust salt from the lagoon was prepared for growth medium for D. salina. Borowitzka and Borowitzka (1990) suggested that Heteramoeba sp. and Cladotricha sigmoidea would usually found contaminated in Dunaliella pond when salinity was dropped. Moulton et al. (1987a) reported in competition between D. salina and D. viridis in Dunaliella culture pond in Australia. They suggested that D. viridis has faster growth rate but there was no data on competitive advantage over D. salina during semicontinuous harvesting. Thus, contamination controlling method was very important for commercial production of D. salina.

In order to control protozoa population in the pond, high salt concentration (more than 25% NaCl) was recommended by Borowitzka and Borowitzka (1990). The method which can inhibit D. viridis population in Dunaliella pond may be done by maintaining the low nitrate concentration (about 0.1 g/l or 10% of nitrate in J/1 medium). This is because D. salina could grow better in low level of nitrate than D. viridis (Borowitzka and Borowitzka, 1988b). The nitrate concentration must be carefully monitored and maintained within the proper amount throughout culture duration.

In order to harvest the algal product, several harvesting methods were proposed and patented, for example, filtration using diatomaceous earth or the use of some behavior including phototactic behavior and hydrophobicity behavior (Borowitzka and

Borowitzka, 1990). However, the combination method especially by flocculation and followed by centrifugation may be a suitable harvesting technique for the commercial production. The result of the current experiment showed that D. salina flocculation by aluminum sulphate was satisfactory. Unfortunately, Aluminum sulphate is not safe enough for food and there was previous report on growth rate reduction by alumina flocculation (Cordero et al., 1990) in semi-continuous harvesting. Therefore, flocculation by others flocculant such as Ferric Chloride (Boussiba et al., 1988) and non-toxic flocculant chitosan (Promjaroen, 1992) should be considered.

It is necessary to remove salt from dried algae because D. salina is cultured in a very high salinity medium. Dried algae from this experiment contained a large amount of ash content (more than 50% ash (Table 5)) which consisted of mainly salt. Therefore, during centrifugation, concentrate algae must be washed with freshwater to remove salt until residual ash was reduced less than 20 percent. In addition, It was found that, different drying method could affected the quality of products. The β-carotene content of freeze dried D. salina (5% AFDW) was higher than the 70°C oven dried (2% AFDW). Seshadri et al. (1991) also reported on β-carotene loss during drying process of Spirulina. They suggested that the losses of β-carotene during drying process could be minimized by lowering the drying temperature and by the use of antioxidants. The bigger drying particle size especially on granular flakes size could maintain the higher β-carotene content than the smaller size in same storage day. Dry alga must be protected from light and minimum access for air/oxygen by using a packed container.

It is necessary to improve the biomass productivity, β-carotene content, and at the same time reducing the production cost by selecting better strains. Improving culture medium and developing the proper culture systems (extensive to intensive outdoor culture or even photobioreactor) are also necessary. The experiment should be done throughout the year to evaluate the annual productivity before scaling up to a commercial project. At the same time, harvesting method and extraction process should be developed and improved for the commercial culture of D. salina in the future.