EGYPTIAN JOURNAL OF AQUATIC RESEARCH 1687-4285
VOL. 31, SPECIAL ISSUE, 2005: 341-351.

USAGE LAKE QUAROUN WATER TO CULTIVATION OF *DUNALIELLA SALINA* AS BIOTECHNOLOGY IN FAYOUM, EGYPT

DARDIR, A.A. AND ABD EL-TAWAB, A.

The Egyptian Salts &minerals Co. EMISAL Egypt

Key words: Lake Quaroun, Cultivation, Biotechnology, β-carotene, Dunaliella salina.

ABSTRACT

As part of development for the mass cultivation of *Dunaliella salina* with high *β-carotene* content, *Dunnaleilla salina* was cultivated as a model organism in outdoor ponds at Emisal Co. (fayoum area). The objective was the optimization of operating condition (temperature, aeration rate, sunlight, brine quality, and biomass density) production of active biomass was optimal at temperature less than 35 ^{o}C, while higher temperature tended to reduce motility and, eventually, growth also for brine quality, where brine has no color, low COD value and low calcium sulphate or carbonate content is good quality for cultivation of *Dunaliella salina* .

INTRODUCTION

The unicellular green alga *Dunaliella* has attracted investigators of various scientific backgrounds since the discovery of the alga at the middle of the l9th century. *Dunaliella* is probably the most halo tolerant eukaryotic organism known and occurs in a wide range of marine habitats, including oceans, brine lakes, salt marshes, and salt ditches near the sea (Ban-Amotz and Avron 1989).

It is found predominantly in water bodies with salt concentrations exceeding 10%, and proliferates in concentrated saline lakes such as the Dead Sea in Israel (Ban-Amotz and Avron 1989) and the northern arm of the Great Salt Lake in Utah (Goldman 1979), where the salt concentration often reaches the saturation level. The early ecological and taxonomic descriptions of *Dunaliella* were followed by studies aimed at the elucidation of the mechanisms that enable the alga to cope with harsh environments, e.g., high saline niches deficient in nutrients, extreme temperatures and pH, and high intensity solar irradiance (Stein 1975).

In the last 25 years, studies of Dunaliella have concerned around several major topics such as:

1. Carotene production

In hypersaline lakes, which are generally low in nitrogen supply and exposed to high solar irradiance, the *Dunaliella* strains that predominate are often orange-red rather than green, due to massive accumulation of *β-carotene*. This property makes these alga strains an excellent model for the elucidation of the carotenoids biosynthetic pathway and its regulation in photosynthetic organisms (Studio Di Ingegnerie 2000).

2. Biotechnology

The unique biosynthetic capacities of *Dunaliella* make it worthwhile to cultivate the alga commercially. Growth in large-scale outdoor ponds is used to produce a mass of dry algal meal enriched in *β-carotene*, or a concentrated algal *β-carotene* solution in oil.

These two products are already being commercially distributed in the special health food, pharmaceutical, and food-coloring markets**.**

Antioxidants, such as *β-carotene*, have been proposed to act as cancer-preventive agents because of their ability to prevent free radicals (Asheley and Michelle1990).

The tested algae is *Dunaliella salina*. It is unicellular and belongs to the class Chlorophyceae and the order Volvocales (Cresswell *et al.* 1989). It is found naturally in many aquatic marine habits containing more than 10% salt, e.g., concentrated Quaron lake water. This lake is hypersaline, the *Dunaliella* strain which predominates in this lake is *Dunaliella salina* plate (1). It is ovoid, motile and halotolerant via an osmoregulation mechanism. It is of a commercial interest because it accumulates massive amounts of the precise product *β-carotene*. Lacking a cell wall but a mucus surface coat, *Dunnaleilla salina* responds easily to external forces by different mechanisms' mainly by secreting *β-carotene* and glycerol. It perforates only in direct sunlight. The present investigation was carried out in door, unless otherwise stated.

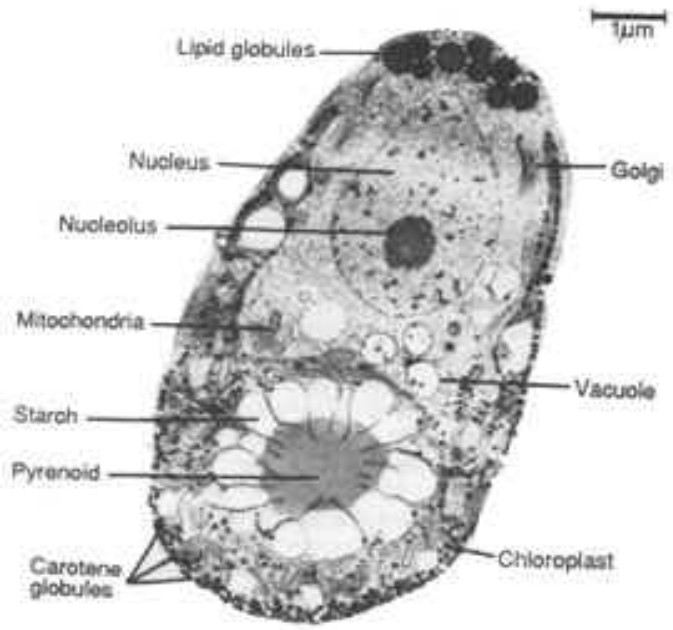

Plate (1): The green alga "*Dunaliella Salina* Teodorescu"

AIM OF THE EXPERIMENT

Commercial production of *D. salina* has been being carried out in SRI, China. But the problems which are being faced now are the short period of time for production because of the rainfall in summer and cold weather in winter and low sunlight intensity which result in low productivity. The experiment we are doing now here in Emisal, Fayoum is to find out whether the conditions here in Emisal including brine, weather, sunlight intensity etc. are suitable for growth of *D.salina* and the possibility of commercial production of *D. salina* in the future.

MATERIAL AND METHODS

1 Cultivation of D. salina in big flasks, bottles and tanks.

To cultivate *D. salina* in outdoor pond, it is necessary to obtain an enough volume of algae culture by cultivation of *D. salina* in big flasks, bottles and tanks.

1.1 Preparation of medium

Screened and boiled brine from concentration pond 3 plate (2) after dilution the total dissolved salts to 160 g/l with tap water in order to get rid of contamination in the brine. After the boiled brine cooled down to room temperature, chemicals were added with final concentration of medium reaching to the following levels:

UREA	0.2 mM
KH_2PO_4	0.2 mM
Fe-EDTA	2.0 μM
$NaHCO_3$	2.0 mM

1.2 About 3litres of *D. salina* culture with cell No. of 0.15×10^6/ ml. was brought from SRI, China. Volumetric cell No. was counted and growth rate was calculated everyday. The culture was diluted with proper amount of medium according to the growth rate to maintain the volumetric cell No. at about 0.2×10^6 / ml.

1.3 To keep a better condition for the growth of *D. salina*, big flasks, bottles and tanks were kept under net shade every morning and moved to a cool place while air temperature reached to 35 ^oC. In the afternoon when the air temperature decreased to 35 ^oC they were moved from the cool place to under the sunlight. And they were shaken once about every hour during the daytime. Sometimes tap water was added to the tanks to maintain the salinity at 160 g/l.

1.4 Cultivation of *D. salina* in the big flasks, bottles and tanks started on 24th August, ended on 19th September with about 90litres of *D. salina* culture finally.

2 Pond construction (Plate 2)

Four culture ponds plate (2) were constructed completely. Areas of culture pond1, culture pond 2, coulture pond 3 and culture pond 4 (short for c-pond 1, c-pond 2, c-pond 3 and c-pond 4) are 12.1, 13.2, 19.2 and 19.2 m^2, respectively. C-pond 1 and 2 were built up with earth and then were lined with plastic film that is 0.28 mm in thickness. A net shade was built up over c-pond2 to reduce the sunlight intensity while volumetric cell No. of culture is low at the beginning of the outdoor cultivation in pond. For c-pond 3 and 4, wood stands were used instead of earth to support the pond of plastic film with a thickness of 0.12 mm. To prevent dogs from approaching the ponds, a fence was built up around the ponds area.

Plate (2): Ponds construction: under shade c-pond 2, beside shade c-pond1 and the other c-pond 3 [Emisal].

3 Cultivation in outdoor ponds

3.1 Brine from concentration pond 3(160 g/l) and 4 (330 g/l), mother liquor (200 g/l), Alexandria (200 g/l) and Port Said (200 g/l) were tested respectively to cultivate *D. salina* in outdoor ponds. Brines was sterilized after screening with 500 ppm NaClO solution (≈10 %) in c-pond witn net shade. Salinity and depth of the brine in c- pond were adjusted to about 16 Bé and 11 cm in advance. Chemicals were added to the brine in c-pond after sterilization for about 24 hrs with final concentration of medium reaching to the same levels as previously described in 1.1.

3.2 About 90L of *D. salina* culture with 0.3-0.6 × 10^6 / ml in cell number was added into the fresh medium in c-pond with a final salinity of 15.5-18 Bé and a depth of 11cm. Culture in one c-pond under net shade was moved to another c-pond without net shade hence the cells number reached to about 0.2 × 10^6 / ml. Cell No., salinity, depth, air max and min temperature, brine max and min temperature, pH etc. were measured every morning and recorded on the tables. Tap water was sometimes added to adjust the salinity of the culture. The culture was stirred as many times as possible. Chemicals added sometimes and carotene content measured while cell No. in culture reached to more than 0.1 × 10^6 / ml were recorded on the same table. Cell No. and carotene content in the culture were shown on figures. Method and measurement of total dissolved salts, cell number and β- carotene as fallow.

- Total dissolved salts measurement:

Total dissolved salts determine by taking of known volume from Sample then evaporation in oven at 110 ^oC where the final weight calculated as g / l after multiple by dilution factor (Emisal Laboratories).

• Cell number counting

Count by taking one drop after mixing well, then put in chamber cell counter, then under biological microscope at 100X count any square or average for 2, 3 or 4 squares.(Distric Laboratory part 2).

• β- carotene measurement

β- carotene was extracted by 90% acetone and was measured the absorbance at 450 nm by Perkin Elmer Lambda 2 Spectrometer after evaluate standard β- carotene curve . (Finar Volume 2 1975)

RESULTS

1. Pond 3 (Fig. 1)

D. salina grew fas in the medium prepared with brine from concentration pond 3 at the beginning in c-pond under net shade with 40% of the sunlight. Volumetric cell No. increased from 0.029×10^6 to 0.17×10^6 / ml within four days. *Carotene* content increased rapidly from 7.4 to 10.6 mg/L after transferring to c-pond under full sunlight for one day. And then cell No. and carotene content rose slowly and gradually with the growth time. Seven days after movement to c-pond under full sunlight, cell No. and *carotene* content rose from 0.16×10^6 /ml and 7.4 mg/L to 0.46×10^6 /ml and 16 mg/l respectively. Salinity increased by evaporation from 160 to 180 g/l after movement of the culture under the full sunlight.

2 Mother liquor (Fig. 2)

D. salina grew fast in the brine prepared with mother liquor at the beginning in c-pond under net shade with 40% of the sunlight. Volumetric cell No. increased from 0.038×10^6 to 0.24×10^6 / ml within first four days, and then cell number rose slowly and gradually with the growth time and reached to 0.6×10^6 / ml after transferring to c-pond under the sunlight for ten days. *Carotene* content increased slowly from 6 to 8 mg/l after transferring to c-pond under the sunlight for four days, and then increased fast from the fifth day on reaching from 8 to 16mg/L within five days. Salinity increased by evaporation from 160 to 200 g/l after movement of the culture under the sunlight.

3 Brine from Alexandria (Fig. 3)

D. salina grew fast in the medium prepared with brine from Alexandria at the beginning in c-pond under net shade with 40% of the sunlight. Volumetric cell No. increased from 0.038×10^6 to 0.235×10^6 / ml within the first four days, and then cell number rose slowly and gradually with the growth time and reached to 0.51×10^6 / ml after transferring to c-pond under the sunlight for nine days. Carotene content increased slowly from 5.7 to 8.7 mg/l after transferring to c-pond under the sunlight for seven days, and then increased fast from the eighth day on reaching from 8.7 to 12.5mg/l within two days. Salinity increased by evaporation from 160 to 200 g/l after movement of the culture under the sunlight.

4 Brine from concentration pond 4 (Fig. 4)

D. salina grow fast in the medium prepared with brine from concentration pond 4 at the beginning in pond under net shade with 40% of the sunlight. Volumetric cell number increased from 0.033 to 0.19×10^6/ ml within four days. And then, however the color of the culture was found abnormal and many cells of small size were observed. So the cultivation with this brine was terminated and started again with fresh medium from concentration pond 4. But the same phenomenon was found again. Neverthless this cultivation was continuted until the cell number reaching from 0.066 to 0.92×10^6/ ml at the end of the experiment. The carotene content reached only to only 3.5 mg/l at the end after 12 days of cultivation under full sunlight.

5 Brine from Port Said (Fig 5)

D. salina-grew slowly in the medium prepared with brine from Port Said at the beginning in the C-pond 4 without net shade and then c-pond with one layer of net. The color of the culture and the cells looked normal with the first three days. And then the

small size of cells and abnormal color of the culture were observed. The cell number was increased from 0.038 to 0.73 × 10^6 / ml within 18 days and carotene content from 1.6 of the sixth days to 5.2 mg/l at the end.

6 Cultivation in flasks with mother liquor and brine from Port Said

To confirm and make sure whether the abnormal phenomenon of the brine from Port Said abovementioned in 5 were due to the brine itself or not, supplementary experiment in four big flasks of 5 L each with mother liquor and brine from Port Said were carried out. Cultivation with each brine was in duplicate. An average of cell number was taken for each brine. At the beginning *D. salina* grew fast in each brine brine within the first five days, with cells number in brine from Port Said from 0.068 to 0.22×10^6 / ml and in mother liquor from 0.086 to 0.214 ×10^6 / ml, respectively. Later on the growth was slow down in both cultures. The carotene content in brine from Port Said and mother liquor reached to 2.7 and 2.8 mg/l at the end of cultivation.

DISCUSSION

The similar results of cultivations with brine from concentration pond 3, mother liquor and brine from Alexandria are observed, which is reflected by the growth and accumulation of carotene. These results are also comparable to that in SRI, China (China 2003) *D. salina* growth and accumulation of *carotene* should have been promoted if there had been a continuous-stirring equipment. However, the brine from Alexandria, at least from El Mex Brines Company, is not suitable for the commercial production of *D. salina* due to its turbidity, red to pink color and high COD, which will adversely affect the growth, the accumulation of carotene and the quality of final product.

The abnormal color and the small size of the cells observed twince in the culture with brine from concentration pond 4 may be due to the brine itself, sharp fluctuation of brine temperature and some unknown reasons.

The abnormal phenomena were also observed in the culture with brine from Port Said, which may be caused by brine itself, sharp fluctuation of culture temperature and mechanical damadge by pumping while cell number was low. However, there was still some culture with brine from Port Said left in c-pond 4 after transferring part of the culture in to c-pond 1. The relatively normal culture color and higher carotene content of the culture remaining in c-pond 4 suggest that the abnormal phenomena of culture with brine from Port Said may not be caused by brine itself but partly by pumping. The supplementary cultivation in four big flasks with mother liquor and brine from Port Said has indicated the similar growth and accumulation of carotene, which further confirms that an abnormal phenomenon with brine from Port Said were not caused by brine itself.

In order to reduce the culture volume for harvesting and increase the carotene content of *D. salina*. Salinity should be increased by evaporation at the later stage of each cultivation. However, when the salinity of the culture with brine from concentration pond 3 increased to 200 g/l. There was a lot of white deposits would suspended in the culture after stirring, which would affect adversely the growth, accumulation of carotene and the quality of final product. Thus, no matter what the deposits are, the brine from concentration pond 3 is not suitable for growing *D. salina*. There were also some white deposits in the culture with mother liquor when the salinity reaching to high level. In regard to culture with brine from Port Said, there some deposits too. But there fewer deposits and much clear culture in c-pond 4 than c-pond 1. Further studies on this issue will be required. In addition, there were a lot of dust from air not only in the c-ponds but also on the net shade and the fence. It is necessary to find a proper solution on this problem.

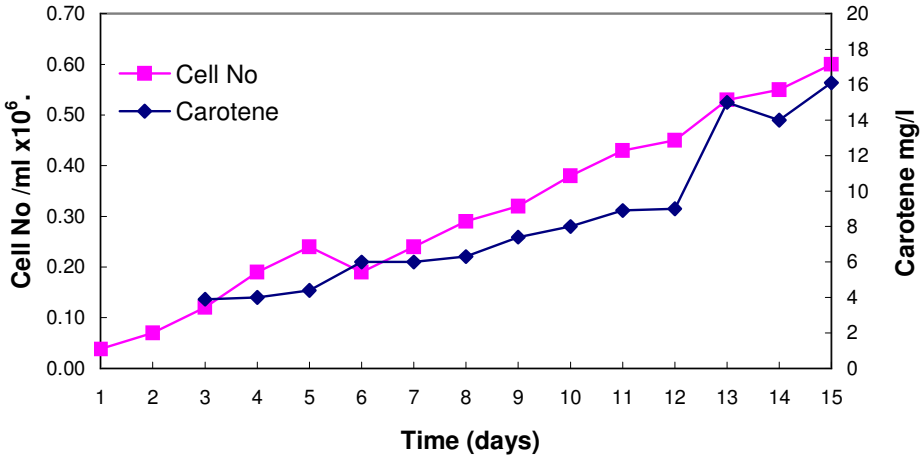

Fig.1. Changes in cell No. and carotene with growth time in medium with brine from Pond 3

1. At day five, the culture was transferred from c-pond under net shade to another c-pond without net shade and diluted with fresh medium.
2. Cell No.:10^6 cells/ml, carotene: mg/L

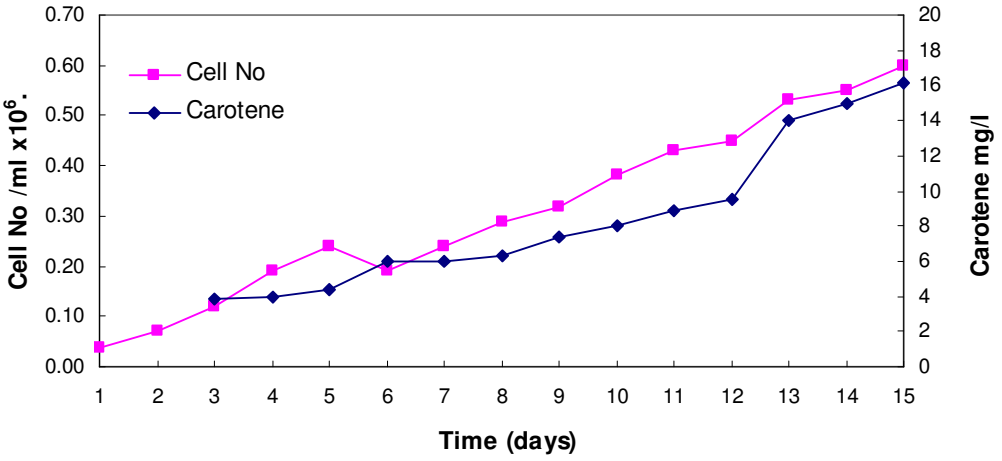

Fig. 2. Changes in cell No. and carotene with growth time in medium with M.L

1. At day five, the culture was transferred from c-pond under net shade to another c-pond without net shade and diluted with fresh medium.
2. Cell No.:10^6 cells/ml, carotene: mg/l

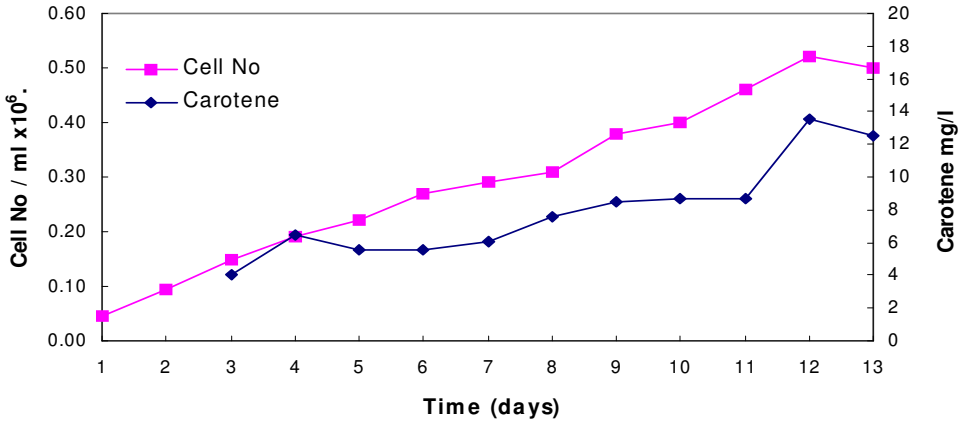

Fig.3. Changes in cell No. and carotene with growth time in medium with brine from Alexandria.

1. At day five, the culture was transferred from c-pond under the net shade to another c-pond without net shade.

2. Cell No.:10^6 cells/ml, carotene: mg/l

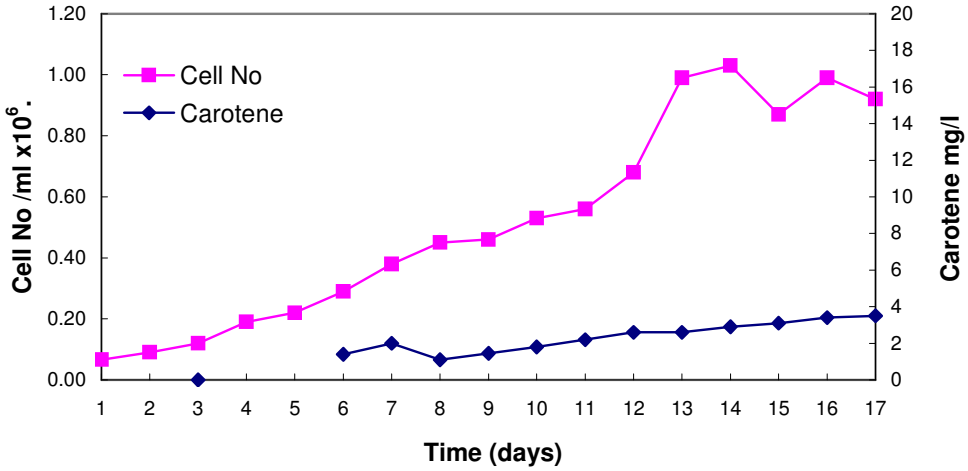

Fig. 4. Changes in cell No. and carotene with growth time in medium with brine from Pond 4

1. At day three, the net shade cut off.

2. Cell No.:10^6 cells/ml, carotene: mg/l

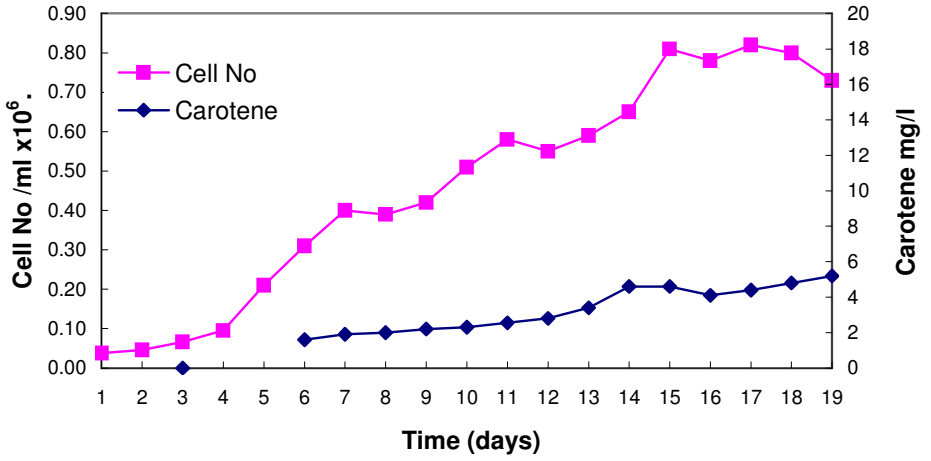

Fig.5. Changes in cell No. and carotene with growth time in medium with bine from Port Said

1. At day nine, the net shade cut off.
2. Cell No.:10^6 cells/ml, carotene: mg/l

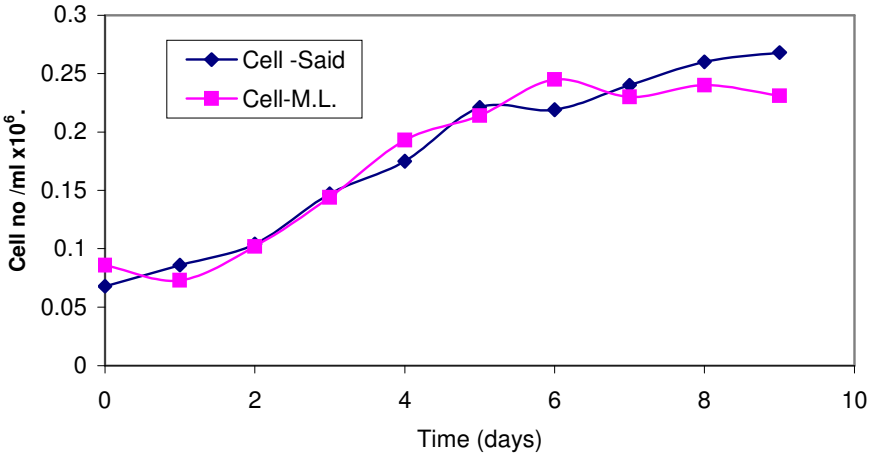

Fig. 6. Changes in cell No. with growth time in media with brines from M.L and Port Said in flasks, Cell No.:10^6 cells/ml

CONCLUSION

Some more supplementary experiments with mother liquor and brine from Port Said carried out in SRI, China, where these Experiments supported this work to applicable the project , these Experiments proved that, the mother liquor of Emisal co. and brine from Port Said are feasible to production of dry algae of *D. salina* .

REFERENCES

1- A.Ben-Amotz and M. Avron : Algal and Cyanobacteria Biotechnology (R.C. Cresswell, T.A.V. Ress & N. Shah, eds.), Longman Scientific and Techenical Press, PP 90-114,1989.

2- Goldman J. C. 1979a. Outdoor algal mass cultures. I. Application. Wat. Res. 13:1-19

3- Handbook of Phycological Methods-Culture Methods and Growth Measurements. Stein J, editor. Cambridge University. Cambridge 1975.

4- Studio Di Ingegnerie Leonardi, StIL Engineering & Research Co. Italy.

5- Asheley R. Kiefer and Michelle L. Slover ,Clarke College 1990 Clarke Dr. Dubuque, IA 52001 Asheley Kiefer @ Clarke. Edu.

6- R.C. Cresswell, T.V.A. Rees and N. Shah, eds." Algal and Cyanobacterial Biotechnology "Longman Scientific and Technical, 1989.

7- The Egyptian Slts &Minerals Co. Emisal,Fayiom.

8- Organic Chemistry Volume 2 I.L. Finar 5th Edition, 1975.

9- District Laboratory Practice in Tropical Counties, part 2, Monica Cheesbrough, 2000.

10- China National Salt Corporation, SRI Co. (Co-operation Report Study with Emisal 2003).