A COMPARATIVE STUDY ON BIOCHEMICAL CHARACTERIZATION OF *DUNALIELLA SALINA* TREATED WITH ETHYL METHANE SULFONATE

Chanchal Lata*[1], Gajendra Pal Singh[2]
Algal Biotechnology Lab, Department of Botany,
University of Rajasthan, Jaipur-302004 (Rajasthan) India.

Abstract

Dunaliella salina is single green algae, isolated from Sambhar Salt Lake, which has very high percentage of carotenoids. The effect of different concentration of mutagen in the form of ethyl methane sulfonate was studied by rapid addition and having treatment for 1hr in dark and its effect on various bio-pigments viz. total chlorophyll, carotenoids and protein contents were determined. Further it was observed that chlorophyll content in treated cultures were decreased as compare to untreated (control) cultures. Maximum carotenoids content was observed at 0.005% EMS on 6th day through rapid addition of EMS. Maximum protein content was observed at 0.04% on 8th day while in cultures kept in dark showed slight increase in chlorophyll as compare to rapid addition of EMS.

***Keywords*: *D.salina, Chlorophyceae*, Bio pigments, EMS, Protein, SDS-PAGE, Chlorophyll and Carotenoid**.

I. INTRODUCTION

Dunaliella salina is a hyper-halo tolerant green microalgae found in high densities in saline lakes (Brock, 1975 and Schlipalius, 1991) and able to tolerate NaCl concentrations (0.2% to 35 %) (Farhat et al., 2011). *D. salina* is richest natural source of *β*- carotene (Farhat et al., 2011). The β- carotene is used as natural food coloring agent in processed foods and cosmetics (Dufossé et al., 2005). One of the most valuable characteristic of *D. salina* is its antioxidant property. Previous researchers had demonstrated that this algae can protect cornea from UVB-induced damage (Tsai et al., 2012). An extract of *D. salina* decreases the human lung cancer cell proliferation by 48% by inducing cell death (Sheu et al., 2008) and extract is also effective against skin cancer cells(Emtyazjoo et al., 2012). Previous researchers had reported that these algae reduce narrowing of the arteries (Sheu et al., 2010) and protect against atherosclerosis (Karppi et al., 2013) through decreasing oxidation and increasing antioxidant activity. Markers of antioxidant activity were greatly increased in rat by receiving its β- carotene (Murthy et al., 2005). *D. salina* as algae-based vaccine will be a breakthrough in resolving aquaculture problems such as viral disease in crustacean (Feng et al., 2014).

The primary objective of the mutation is to enlarge the frequency and spectrum of viable mutations, as an approach towards directed mutagenesis. Chemical mutagenesis can be used not only to loss- or gain-of-function mutants but also to understand the protein function.

Ethyl methane sulfonate (EMS) is mono-functional ethylating agent that has been found to be mutagenic induce mispairing and base changes. EMS can mimic nitrogen base in normal DNA but it cannot couple during DNA replication (Sega, 1984). It has also been shown to be carcinogenic in mammals. Genetic data obtained using microorganisms suggest that EMS may produce both GC to AT and AT to GC transition mutations and can cause base-pair insertions or deletions as well as more extensive intragenic deletions (Sega, 1984). Some reports revealed that EMS gradually hydrolyses DNA form the deoxyribose on backbone (mostly the *N*-7 position of guanine) and leaving behind an apurinic (or possibly an apyrimidinic) site that is unstable and can lead to single-strand breakage of the DNA and causing chromosome breakage (Sega, 1984)

Many of the damaging effects of chemical mutagens or physical factors are reduced through defense mechanisms. (ib Bhattacharya, 2011, Boran et al., 2017 and Słoczyńska et al., 2014) Production of anti-mutagenic agent in the cell is a step of defense mechanisms. So, chemical mutagens induces and improve the quantity of nutritionally useful products involved in defense mechanism in organisms that are important for human welfare. There are many studies reported chemical mutagens induce and improve cell function(James et al., 2013, El-Nashar et al., 2015 Kishk et al., 2016)

There are many studies reported by scientists which favours that EMS affects carotenoids, chlorophyll and proteins. (Kumar et al., 1974, Hairui et al., 1997, Doan et al., 2012, Tripathi et al., 2001, Huesemann et al., 2009, Chamovitz et al., 1993, Liu et al., 2010, Hu et al., 2008 and Jin et al., 2003) In a different study EMS produces pollution-tolerant strains, (Kumar et al., 1974) enhances intracellular lipid (Doan et al., 2012) and improves biomass productivities (Tripathi et al., 2001and Huesemann et al., 2009). Overproduction of carotenoid in *Haematococcus* mutants while reducing cell mortality during stress-induced carotenogenesis was reported. (Hu et al., 2008) It has been reported mutant of alga *Dunaliella salina* constitutively accumulates high carotenoid under all growth conditions (Jin et al., 2003).

The present study was aimed to investigate the possibility of effect of EMS on proteins and bio pigments in *D. salina* through induce mutagenesis.

II. MATERIALS AND METHODS

Dunaliella salina was isolated from Sambhar Lake. Isolation and purification was made by dilution, plating technique. Culture were grown and maintained under ASWM (Lata et al., 2016)at 26±2^{0}C temperature under cyclic fluorescent illumination (12 hrs dark: 12 hrs light) of 2500 lux.

We design two type of experimental arrangements to ensuring the better effect of EMS on chlorophyll, carotenoid and proteins content of *D. salina*

1. *Rapid addition of EMS*

Uni-algal cultures were added to 500ml conical flasks containing 250ml artificial sea water medium (ASWM) with different concentration of EMS i.e. 0.005%, 0.01%, 0.02%, 0.04% and 0.08% for 12 days duration along with controlled culture conditions. The cultures were grown in EMS treated media at 26±2^{0}C under cyclic fluorescent illumination (12 hrs dark: 12 hrs light) of 2500 lux. Non-treated *D. salina* cultures in artificial sea water media were used as control. In order to find out effective concentration of EMS mutagen and effect on pigmentation, protein content during rapid addition of EMS culture were observed on 2nd ,4th, 6th,8th,10th, and 12th days for chlorophyll, carotenoid and protein content.

2. *1 hour treatment of EMS in dark*

Uni-algal cultures were centrifuged at 5000 rpm for 5 min, centrifuged cells were treated with varied concentrations of EMS i.e. 0.005%, 0.01%, 0.02%, 0.04% and 0.08%. 5 ml of EMS solution was used for each concentration for soaking. The cells were treated with EMS of specified concentrations for 1 hour in dark under controlled conditions with intermittent shaking. After 1 hour, the EMS solution was removed through centrifuge and cells were thoroughly washed in ASWM for 4 times to remove the EMS residues using centrifuge. After 4 times washing, treated algal cells were added to 500ml conical flask containing 250ml artificial sea water media (ASWM) for 12 days duration along with controlled culture conditions. The cultures were grown at 26±2^{0}C temperature under cyclic fluorescent illumination (12 hrs dark: 12 hrs light) of 2500 lux. Non-treated *D. salina* cultures in artificial sea water media were used as control. In order to find out effective concentration of EMS mutagen and effect on pigmentation, protein production during 1 hour treatment of EMS in dark culture were observed on 2nd ,4th, 6th,8th,10th, and 12th days for chlorophyll, carotenoid and protein content.

Estimation of Bio pigments

- Total chlorophyll was determined by the method of Arnon. (Arnon., 1949)
- Total Carotenoid was estimated by method of Mahadevan and Sridhar (Mahadevan & Shridhar).

Estimation of protein content

- For quantitative estimation of Protein, protocol of Osborne's was followed (Osborne, 1962) and the quantity of total protein content was estimated by Lowry's method (Lowry, 1951).

SDS PAGE analysis

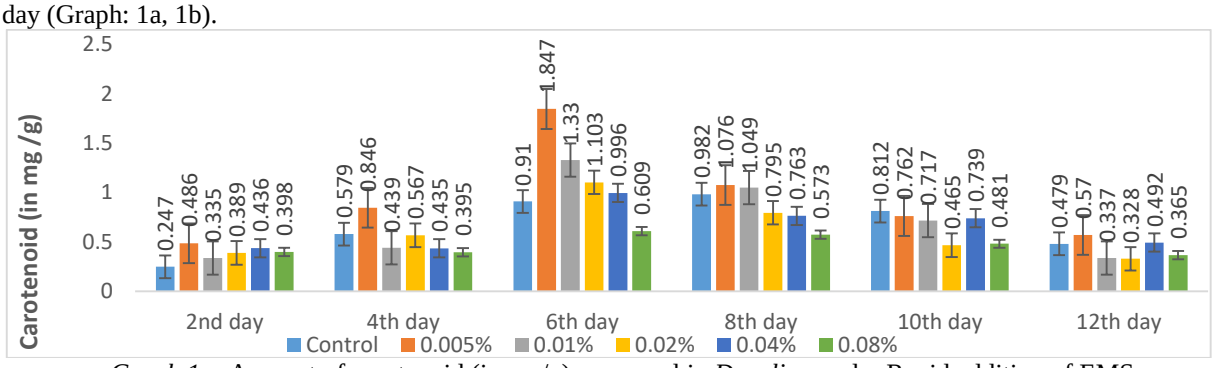
The effect of different concentration of EMS on protein profile was determined through SDS-PAGE. Cultures exhibited highest growth parameters, bio pigments, protein content were followed for SDS-PAGE. Protein extraction was carried out according to Naushad (Turi et al., 2010). Electrophoresis was carried out according Laemmli's method (Laemmli, 1970).

In order to score and preserve banding pattern, the gel was subjected to image scanning using BIO-RED GS-700 Imaging Densitometer (USA) and the protein profiles were obtained for each variety. The band were designated on the basis of their molecular weight, for this purpose molecular weight marker ranging from 14.4kDa to 116.0kDa was loaded simultaneously with samples. The distance run by amplified fragment, from the well translated to molecular weight with reference to protein molecular weight marker. The presence of each band was scored as (+) plus and it absence as minus (-)

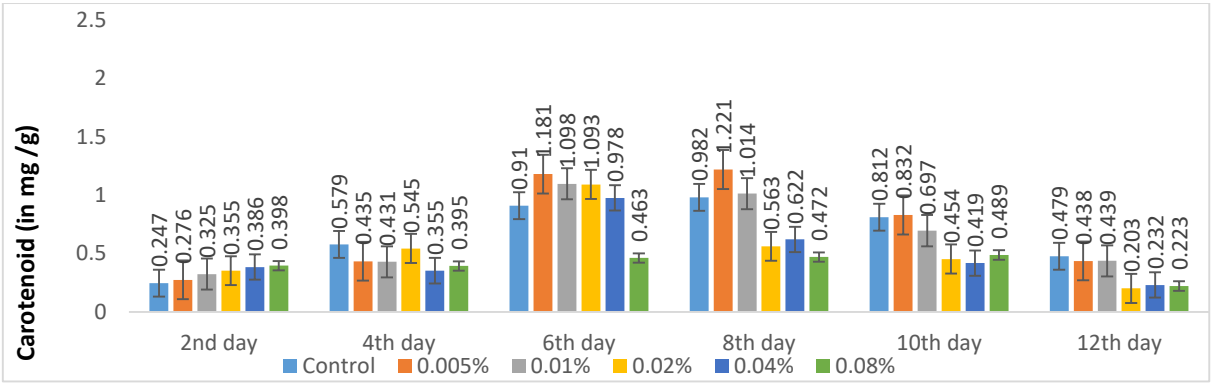
III. RESULTS AND OBSERVATIONS

Effect of different concentration of EMS on carotenoid

Overall maximum amount of carotenoids was observed in rapid addition of EMS at 0.005% dose and minimum carotenoids was observed in 1 hour treatment of EMS in dark at 0.08% dose (Graph: 1a, 1b). Carotenoid increased in both treatments as compare to control but lower in quantity in 1 hour treatment of EMS in dark. Overall carotenoid was found highest mostly on 6th day (Graph: 1a, 1b).



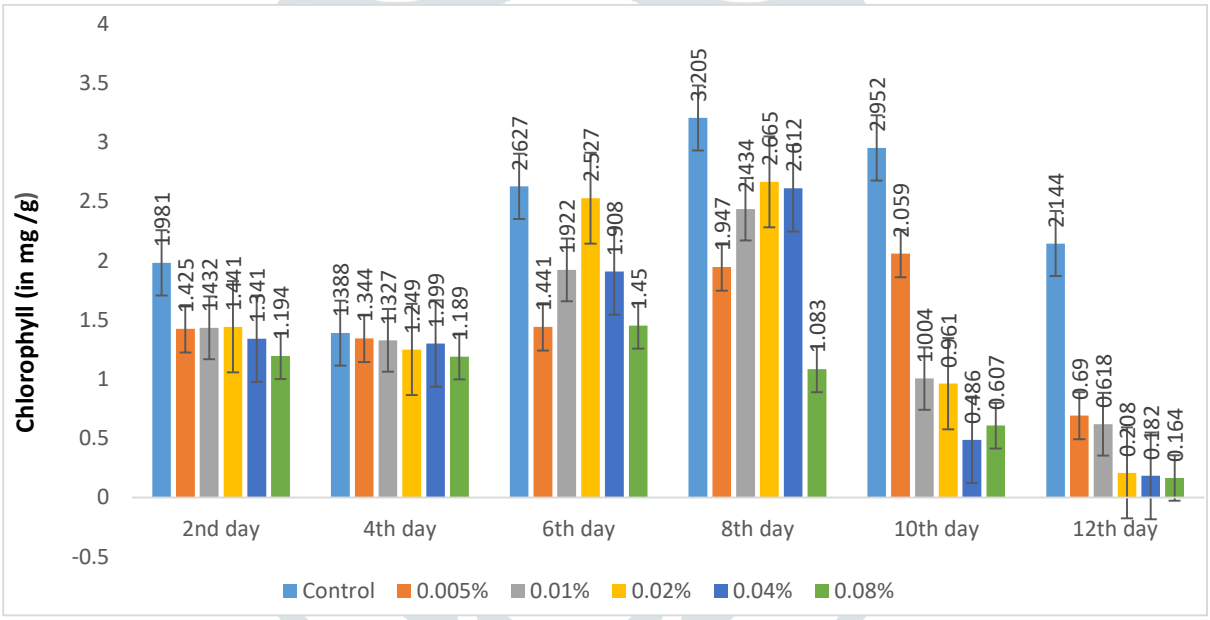
Graph 1a: Amount of carotenoid (in mg/g) measured in *D. salina* under Rapid addition of EMS.



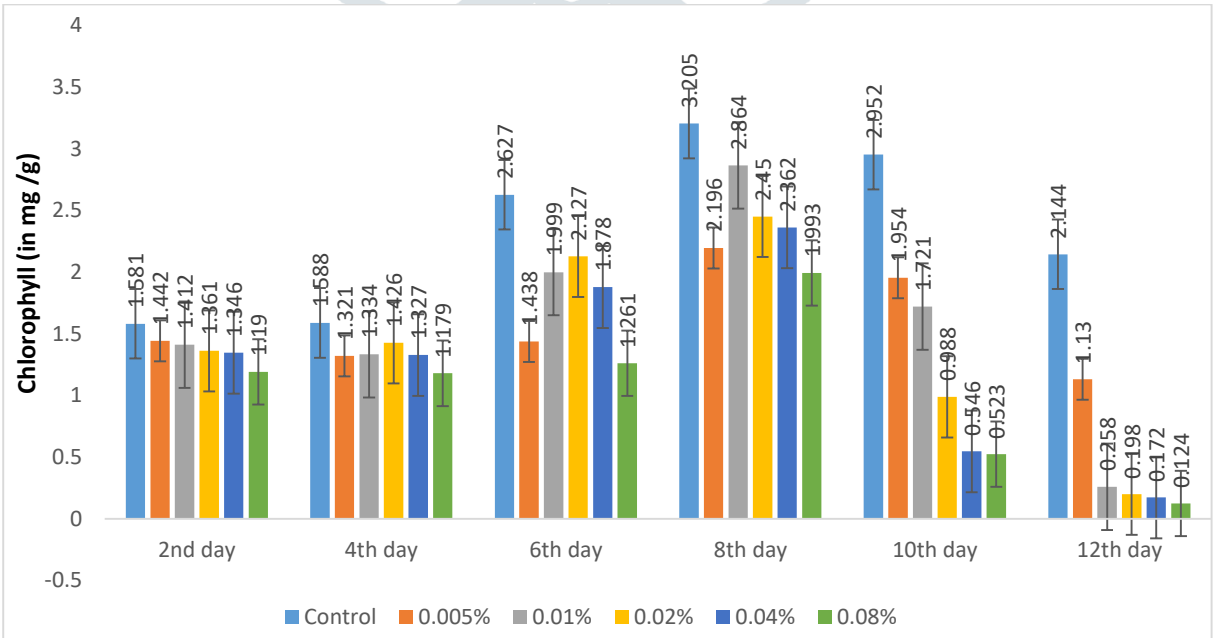
Graph 1b: Amount of carotenoid (in mg/g) measured in *D. salina* under 1 hour treatment of EMS in dark.

Effect of different concentration of EMS on Chlorophyll

Overall Maximum chlorophyll was observed in control on 8th day and minimum was observed on 12th day in 1 hour treatment of EMS in dark at 0.08% dose. Chlorophyll was found slightly high in 1 hour treatment of EMS in dark as compared to rapid addition of EMS. Overall chlorophyll was found maximum mostly on 8th day (Graph: 2a, 2b).



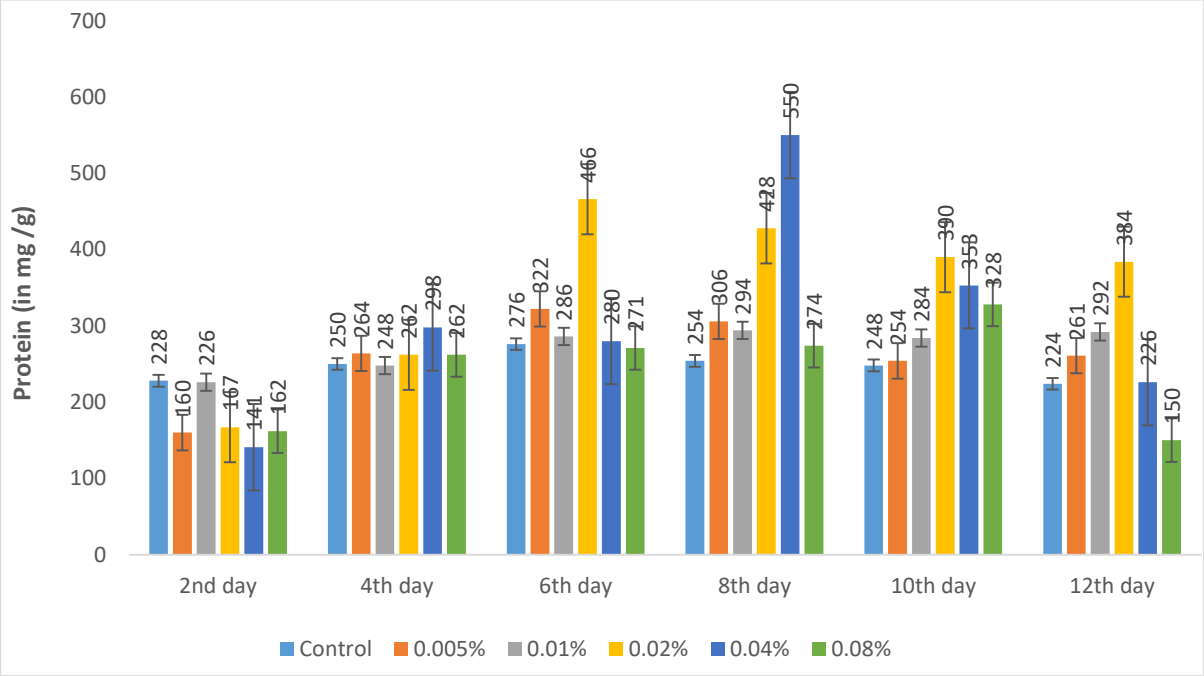
Graph 2a: Amount of chlorophyll (in mg/g) measured in *D. salina* under Rapid addition of EMS.



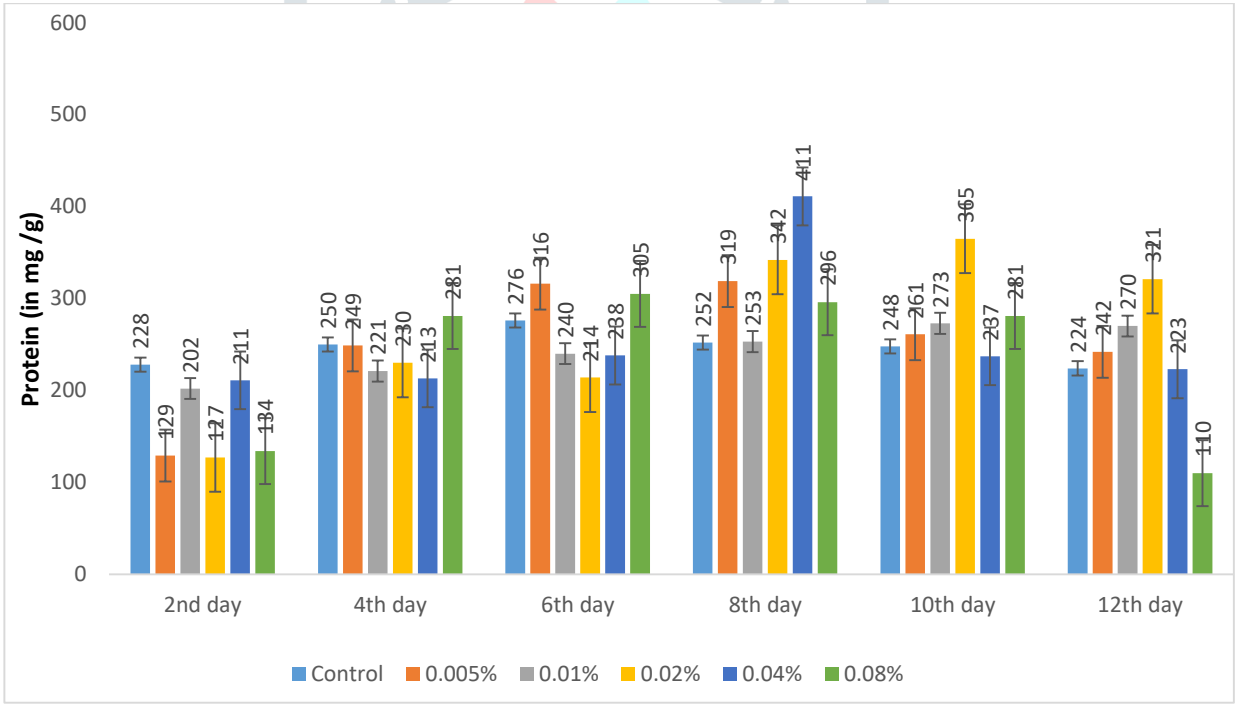
Graph 2b: Amount of chlorophyll (in mg/g) measured in *D. salina* under 1 hour treatment of EMS in dark.

Effect of different concentration of EMS on Protein content

Overall highest protein was observed in rapid addition of EMS at 0.04% dose on 8th day. Least was found in 1 hour treatment of EMS in Dark at 0.08% dose. Protein was found highest mostly on 8th day (Graph: 3a, 3b)



Graph 3a: Amount of proteins (in mg/g) measured in *D. salina* under Rapid addition of EMS.



Graph 3b: Amount of proteins (in mg/g) measured in *D. salina* under 1hour treatment of EMS in dark.

Effect of different concentration of EMS on SDS- PAGE Analysis

Culture treated by rapid addition of EMS were found the best in all aspect (such as bio pigments and protein quantity) as compared to culture treated with EMS for 1hour in Dark. So, finally Culture treated by rapid addition of EMS were analysed for protein profiling through SDS-PAGE. In each gel a wide range molecular weight marker (Fisher) was included. M: Marker in kDa, L1: control, L2: 0.005%, L3:0.01%, L4:0.02%, L5:0.04%, L6:0.08% was different concentration of EMS resolved in SDS PAGE. After staining and de-staining, gel was scanned and photographs were taken. As shown in (Fig: 1) polypeptide profile of extracted protein in SDS-PAGE, there were some newly expressed and some highly expressed polypeptide bands.

As compared to control, there were some newly expressed polypeptide bands such as P1 in 0.04%EMS treated *D. salina* (As shown in in L5 of Fig. 1) and P2, P3 in 0.02%EMS treated *D. salina* (As shown in in L4 of Fig. 1).

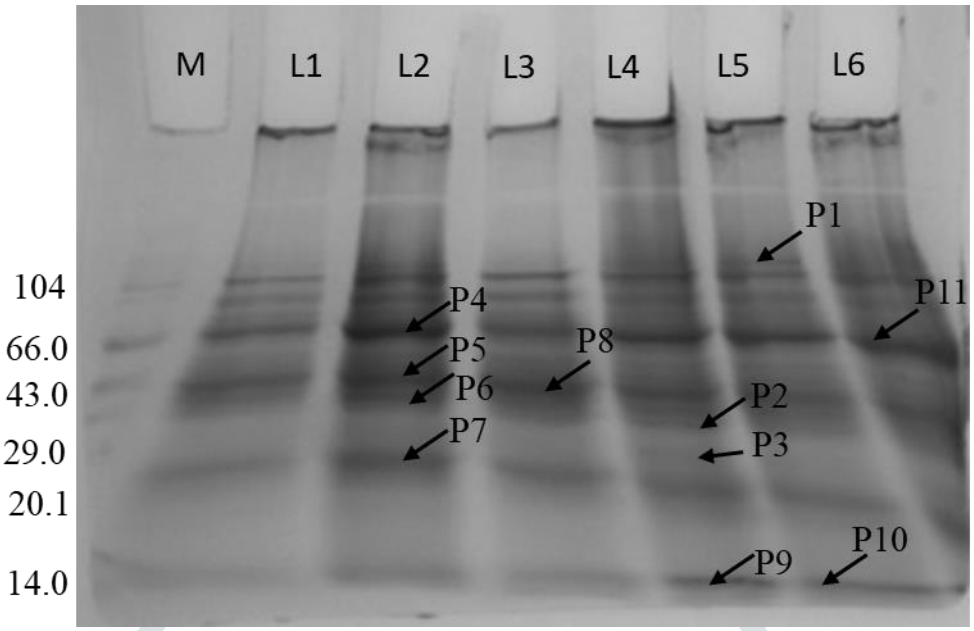


Fig 1: Coomassie brilliant blue stained polypeptide profile of extracted protein separated by SDS-PAGE. The protein were extracted from *D. salina* under EMS stress treatment. M: Marker in kDa, L1: Control, L2: 0.005%, L3:0.01%, L4:0.02%, L5:0.04%, L6:0.08% of EMS treated culture.

As compared to control, there were some highly expressed polypeptide bands such as P4, P5, P6 and P7 in 0.005%EMS treated *D. salina*, (As shown in in L2 of Fig. 1), P8 in 0.01%EMS treated *D. salina*, (As shown in in L3 of Fig. 1), P9 in 0.02%EMS treated *D. salina* (As shown in in L4 of Fig. 1), P10 in 0.04%EMS treated D. salina (As shown in in L5 of Fig. 1) and P11 in 0.08%EMS treated *D. salina*, (As shown in in L6 of Fig. 1).

All the band were designated between 14.4 kDa to 116.kDa on the basis of their molecular weight. The distance run by amplified fragment, from the well translated to molecular weight with reference to protein molecular weight marker the presence of each band was scored as (+) plus and it absence as minus(-).

IV. DISCUSSION

The potential roles of EMS in terms of their comparative advantage over their wild type (control) and mutants (treated) for the production of carotenoid with respect to their potential utilization by the algal biotechnology industry are discussed. However a better understanding of the interrelationship between stress and carotenoid overproduction will facilitate an innovative idea for production of specific carotenoids and other products in *D. salina* and in related organisms.

The protocol of EMS treatment having 1hour in dark is not an effective method to induce carotenoid, chlorophyll and protein as compared to control culture and to cultures treated by rapid addition of EMS also. This may be due to loss in viability by mechanical damage induces by 4 time washing of *D. salina* through centrifugation.

Overall biomass productivity of mutants were higher than control and achieving the highest volumetric productivity of carotenoid (1.8 mg g^{-1} d^{-1}). Carotenoid content was increased significantly as compared to control due to stress. Low concentration of EMS causes significantly low stress which supports cell stability to induce a high amount of total carotenoid productivity. It has been reported that overproduction of carotenoid in *Haematococcus* mutants reduced cell mortality during stress-induced carotenogenesis (Hu et al., 2008).

Hence biomass productivity of mutants were substantially high in very low concentration of EMS (0.005% to 0.04%) than to high concentration of EMS. The mutants thus obtained showed enhanced (2.2–3.2-fold) carotenoid accumulation at very low concentration as compare to control. It has been also reported that *Dunaliella* grown under stress conditions produce higher carotenoid 14% of dry weight of cell (Emeish, 2012).

Overall photosynthetic productivity as measured by chlorophyll content of mutants were low as compare to control achieving the highest volumetric productivity of chlorophyll (3.2 mg g^{-1} d^{-1}) on 8th day. Lowest chlorophyll content (0.124 mg g^{-1} d^{-1}) was found on12^{th} day of experimental period in culture treated 1hour with 0.08%EMS in dark treatment. Photosynthetic productivity were substantially low in high concentration of EMS (0.01% to 0.08%). Here photosynthesis is clearly an important target of EMS toxicity.

Hence, the chlorophyll content was decreased and carotenoid was increased compared with the control values and both chlorophyll and carotenoid reduced with increasing EMS concentration. Volumetric productivity of biomass was closely associated with the cellular content of total chlorophyll. Results observed at low concentration of EMS also agreed with the previous findings on *D.salina*. (Abu-Rezq et al., 2010) According to previous findings (May SO) under different type of high stress such as high salinities, high light intensity, in nitrogen or phosphorus deficiency the increase in carotenoid occurs. In such a large quantity of carotenoid water becomes red due to algal colour changed from green to red. Red colour due to a decomposition of chlorophyll content of the algal cells and an increase in the β-carotene content.

High concentration of mutagen can causes abrupt change in protein quantity due to activation and induction of cell division inhibitor protein by high stress as showing in EMS dose 0.04%, protein content was found highest 550 mg g^{-1} d^{-1} among 8th day. When we compare the both protocol with the control with respect to protein content, we found overall Protein content of both treated cultures were high as compare to control. From the day of induction the total protein productivity in 0.005%EMS was found 322 mg g^{-1} d^{-1} (in rapid addition of EMS) on 6th day, whereas it was 316 mg g^{-1} d^{-1} (in 1hour treatment of EMS in dark) on same day, on the other hand it was 276 mg g^{-1} d^{-1} (in Control) on same day. The protein content showed some variation due to synergic effect of time intervals and concentrations of EMS. Increment in protein quantity as compare to control may also support to enhance carotenoind production. It has been reported that a point mutation in the phytoenedesaturase enzyme (*PDS)* gene by chemical mutagen enhance carotenoid production in a strains of the *cyanobacterium Synechococcus sp*. PCC 7942(Chamovitz et al., 1993) and *Chlorella zofingiensis* mutants (Liu et al., 2010). This gene was responsible for desaturation of phytotene.

On the other hand, from the results obtained through SDS-PAGE, it is showed that differential protein expression induced by very low concentration (0.005%) of EMS. differential protein expression is not reduced by EMS hence it is suggested that protein can protect cells against damage induced by very low concentration (0.005%) of EMS and may support in enhance carotenoid production. Statistically, the variations in biochemical analysis due to different concentration of EMS treatment of *D. salina* studied were significant.

Hence when culture treated with very high concentration 0.08 % of EMS, cell experiences excessive stress due to inhibition of cell division ultimately cell not supports carotenoid production. Photosynthesis also gets adversely affected. Hence the chlorophyll content was decreased with increase in concentration of EMS.

These mutants are discussed in terms of its commercial value and potential utilization by the algal biotechnology industry for the production of carotenoid. Moreover, future directions that might further our knowledge in this area are given.

V. CONCLUSION

The mutants thus obtained showed reduced chlorophyll content and enhanced (2.2–3.2-fold) carotenoid accumulation at low concentration in case of rapid addition of EMS. Improvement in the carotenoid content in addition to low concentration of EMS through rapid addition could be a good basis for the exploitation of microalgae as a source of bio pigment.

Mechanical damages reduces bio pigments, and protein production as in 1 hour treatment of EMS in dark protocol.

It was concluded that bio pigments varies with different concentration of chemical mutagen and EMS induce its positive effect for bio pigments production with very low concentration. There are many studies have been reported in supporting our findings. (Kozgar, 2014, Satpute et al., 2012).

VI. ACKNOWLEDGMENTS

The authors are thankful to Head Department of Botany, University of Rajasthan for providing necessary facilities and UGC for providing financial support.

VII. REFERENCES

1. Abu-Rezq T.S., Al-Hooti S. and Jacob D.A., 2010. Optimum culture conditions required for the locally isolated Dunaliella salina. *J. Algal Biomass Utln.*,1(2):12-19.
2. Arnon, D.I. and Wessel, G., 1953. Vanadium as an essential element for green plants. *Nature*, *172*(4388), p.1039.
3. Boran R. and Ugur A., 2017. The mutagenic, antimutagenic and antioxidant properties of Hypericum lydium. *Pharmaceutical biology*, 1; 55(1):402-405.
4. Brock T.D., 1975. Salinity and the ecology of Dunaliella from Great Salt Lake. *Microbiology*, 89(2):285-292.
5. Chamovitz D., Sandmann G. and Hirschberg J., 1993. Molecular and biochemical characterization of herbicide-resistant mutants of cyanobacteria reveals that phytoene desaturation is a rate-limiting step in carotenoid biosynthesis. *Journal of Biological Chemistry*, 268(23):17348-17353.
6. Doan, T.T.Y. and Obbard, J.P., 2012. Enhanced intracellular lipid in Nannochloropsis sp. via random mutagenesis and flow cytometric cell sorting. *Algal Research*, *1*(1):17-21
7. Dufossé L., Galaup P., Yaron A., Arad S.M., Blanc P., Murthy K.N. and Ravishankar G.A., 2005. Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? *Trends in Food Science & Technology*, 16(9):389-406.
8. El-Nashar Y.I. and Shetta N.D., 2015. Mutagenic Effects of Sodium Azide and Diethyl Sulphate on the Growth of Leucaena Trees (Leucaena leucocephala Lam.) Under Field Conditions. *Alexandria Science Exchange*. 36(3):198.
9. Emeish S., 2012. Production of natural β-carotene from Dunaliella living in the Dead Sea. *JJEES*. 4(2):23-27.
10. Emtyazjoo, M., Moghadasi, Z., Rabbani, M., Emtyazjoo, M., Samadi, S. and Mossaffa, N., 2012. Anticancer effect of Dunaliella salina under stress and normal conditions against skin carcinoma cell line A431 in vitro. *Iranian Journal of Fisheries Sciences*, *11*(2):283-293.
11. Farhat, N., Rabhi, M., Falleh, H., Jouini, J., Abdelly, C. and Smaoui, A., 2011. Optimization of salt concentrations for a higher carotenoid production in Dunaliella salina (Chlorophyceae). *Journal of phycology*, *47*(5):1072-1077.
12. Feng S., Feng W., Zhao L., Gu H., Li Q., Shi K., Guo S. and Zhang N., 2014. Preparation of transgenic Dunaliella salina for immunization against white spot syndrome virus in crayfish. *Archives of virology*, 1; 159(3):519-525.

13. Hairui C., Zhiping W. and Bujin X., 1997. The effects of ethyl methane-sulphonate on the growth and morphology of Spirulina platensis. *Journal of Zhejiang Agricultural University*, 23(6):645-648.
14. Hu Z., Li Y., Sommerfeld M., Chen F. and Hu Q, 2008. Enhanced protection against oxidative stress in an astaxanthin-overproduction Haematococcus mutant (Chlorophyceae). *European Journal of Phycology*, 43(4):365-376.
15. Huesemann M.H., Hausmann T.S., Bartha R., Aksoy M., Weissman J.C. and Benemann J.R., 2009. Biomass productivities in wild type and pigment mutant of Cyclotella sp. (Diatom). *Applied biochemistry and biotechnology*, 157(3):507-526.
16. ib Bhattacharya S., 2011. Natural antimutagens: a review. *Research Journal of Medicinal Plant*. 5(2):116-126.
17. James Walter A., Caroline J.C., Caroline J.J.and Anjanapriya S., 2013. Toxic and mutagenic impact of ethidium bromide traces on the growth of Escherichia coli KL226. *Int. J. Curr. Sci*. 8:28-32.
18. Jin E., Feth B. and Melis A., 2003. A mutant of the green alga Dunaliella salina constitutively accumulates zeaxanthin under all growth conditions. *Biotechnology and bioengineering*, 81(1):115-124.
19. Karppi, J., Kurl, S., Ronkainen, K., Kauhanen, J. and Laukkanen, J.A., 2013. Serum carotenoids reduce progression of early atherosclerosis in the carotid artery wall among Eastern Finnish men. *PLoS One*, *8*(5):e64107.
20. Kishk D.A., Abul-Soad A.A., Abbas M.S., El-Shabrawi H.M. Gaber E.S. and El-Deen T.M., 2016. Effect of diethyl sulphate and sodium azide on tolerance of ex vitro banana to salt stress. *International Journal of ChemTech Research*, 9(12): 81-99.
21. Kozgar I.M., 2014. Mutation Breeding in Chickpea: Perspectives and Prospects for Food Security. *Walter de Gruyter GmbH & Co KG*.
22. Kumar H.D., Sharma V. and Bisaria G.P., 1974. Production of pollution-tolerant strains of unicellular algae through mutagenic treatment. I. Ethyl methane sulphonate. *Journal of Basic Microbiology*, 14(6):487-496.
23. Laemmli U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(5259):680.
24. Lata C., Singh G.P., 2016. Effect of Different Culture Media on Growth and Biopigments of *Dunaliella Salina* Isolated From Sambhar Lake. *Elixir Applied Botany*. 97(2016) 42102-42105
25. Liu J., Zhong Y., Sun Z., Huang J., Sandmann G. and Chen F., 2010. One amino acid substitution in phytoene desaturase makes Chlorella zofingiensis resistant to norflurazon and enhances the biosynthesis of astaxanthin. *Planta,*, 232(1):61-67.
26. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *Journal of biological chemistry*, *193*(1), pp.265-275.
27. Mahadevan, A. and Sridhar, R., 1982. Methods in physiological plant pathology.
28. May S.O. The effect of various environmental factors on the growth of a red pigmented Dunaliella species from the Great Salt Lake.
29. Murthy, K.C., Vanitha, A., Rajesha, J., Swamy, M.M., Sowmya, P.R. and Ravishankar, G.A., 2005. In vivo antioxidant activity of carotenoids from Dunaliella salina—a green microalga. *Life Sciences*, *76*(12):1381-1390.
30. Osborne, D.J., 1962. Effect of kinetin on protein & nucleic acid metabolism in Xanthium leaves during senescence. *Plant Physiology*, *37*(5):595.
31. Satpute R.A. and Fultambkar R.V., 2012. Mutagenic effectiveness and efficiency of gamma rays and EMS in soybean (Glycine max (L.) Merrill). *Current Botany*, 2: 3(2).
32. Schlipalius, L., 1991. The extensive commercial cultivation of Dunaliella salina. *Bioresource technology*, *38*(2-3):241-243.
33. Sega G.A., 1984. A review of the genetic effects of ethyl methanesulfonate. *Mutation Research/Reviews in Genetic Toxicology*, 134(2-3):113-142.
34. Sheu, M.J., Cheng, H.C., Chien, Y.C., Chou, P.Y., Huang, G.J., Chen, J.S., Lin, S.Y. and Wu, C.H., 2010. Molecular mechanism of green microalgae, Dunaliella salina, involved in attenuating balloon injury-induced neointimal formation. *British journal of nutrition*, *104*(3): 326-335.
35. Sheu, M.J., Huang, G.J., Wu, C.H., Chen, J.S., Chang, H.Y., Chang, S.J. and Chung, J.G., 2008. Ethanol extract of Dunaliella salina induces cell cycle arrest and apoptosis in A549 human non-small cell lung cancer cells. *In vivo*, *22*(3):369-378.
36. Słoczyńska K., Powroźnik B., Pękala E. and Waszkielewicz A.M., 2014. Antimutagenic compounds and their possible mechanisms of action. *Journal of applied genetics*, 55(2):273-285.
37. Tripathi U., Venkateshwaran G., Sarada R., Ravishankar G.A., 2001. Studies on Haematococcus pluvialis for improved production of astaxanthin by mutagenesis. *World Journal of Microbiology and Biotechnology*, 17(2):143-148.
38. Tsai, C.F., Lu, F.J. and Hsu, Y.W., 2012. Protective effects of Dunaliella salina–a carotenoids-rich alga–against ultraviolet B-induced corneal oxidative damage in mice. *Molecular vision*, *18*:1540.
39. Turi, N.A., Farhatullah, F., Rabbani, M.A.R., Khan, N.U., Akmal, M., Pervaiz, Z.H. and Aslam, M.U., 2010. Study of total seed storage protein in indigenous Brassica species based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). *African Journal of Biotechnology*, *9*(45):7595-7602.