EFFECTS OF ETHIDIUM BROMIDE AS A MUTAGEN ON BIOCHEMICAL PARAMETERS IN *DUNALIELLA SALINA*

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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 ethidium bromide (EtBr) was studied on various biopigments viz. total chlorophyll, carotenoids and Protein contents. Overall maximum amount of carotenoids was observed in culture added with 0.005% treatment level at 6th day. Carotenoid increased at 0.005 and 0.01% treatment and decreased thereafter as comparison to control. Overall Maximum chlorophyll was observed in control on 8th day. Chlorophyll was slightly reduced in EtBr addition at all treatment dose when compared to control as increase in time intervals and dose level. Overall maximum protein was observed in control on 6th day. Among all treatment dose maximum protein content was observed in 0.04% treatment dose at 8th day. The amount decrease thereafter with increase in treatment dose. In SDS PAGE there were some newly expressed polypeptide bands and some highly expressed polypeptide bands and there was one polypeptide band which reduced approximately in all treated culture as compared to control culture.

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

I. INTRODUCTION

D. salina is a hyper-halo tolerant green microalgae found in high densities in saline lakes (Oren, 2005 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 (Borowitzka, 1995).

One of the most valuable characteristic of this alga is its antioxidant property. Previous researchers had demonstrated that 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 to 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).

The primary objective of the mutation is to enlarge the frequency and spectrum of viable mutations, as an approach towards directed mutagenesis (Ando and Montalván, 2001). Chemical mutagenesis can be used not only to loss- or gain-of-function mutants but also to understand the protein function.

Many of the damaging effects of chemical mutagens or physical factors are reduced through defense mechanisms (Doan & Obbard,, 2012). 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.

EtBr is an aromatic compound with a heterocyclic moiety that emits orange-red light at 605 nm when exposed to 285 nm UV light, especially when bound to DNA (30-fold increase). This property determines that one of the more traditional applications of EtBr is to detect DNA from agarose gels. Another use that has been given to EtBr is as a general indicator of associations of DNA-dependent and DNA-independent protein. These associations DNA-dependent protein were selectively inhibited by EtBr in the precipitation reaction with no apparent effect on DNA- independent protein association (Lai and Herr, 1992). Photophysical properties of this fluorescent dye after interaction with DNA were extensively studied by Cosa et al. (2001) and the work of Karapetian et al (1996) revealed at least three types of complexes of EtBr with ds-DNA.

The present study was aimed to investigate the possibility of effect of EtBr on proteins and biopigments 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 (Ben-Amotz and Avron, 1983) at 26±2^{0}C temperature under cyclic fluorescent illumination (12 hrs dark: 12 hrs light) of 2500 lux.

Treatment of EtBr

Uni-algal cultures were added to 500ml conical flasks containing 250ml artificial sea water medium (ASWM) with different concentration of EtBr 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 mutagen treated media at 26±2^{0}C under cyclic fluorescent illumination (12 hrs dark: 12 hrs light) of 2500 lux. In order to find out effective concentration of EtBr mutagen and effect on pigmentation, protein content during continuous treatment of EMS, and 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 and Wessel (1953).
- Total Carotenoid was estimated by method of Mahadevan and Sridhar (1982)

E**stimation of protein content:** For quantitative estimation of Protein, protocol of Osborne's (1962) was followed and the quantity of total protein content was estimated by Lowry's method (see Lowry et al., 1951)

SDS PAGE Analysis:

The effect of different concentration of EtBr 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 (see Turi et al., 2010). Electrophoresis was carried out according to 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

In case of optical density and dry weight all parameters decreased as compared to control. Overall maximum amount of carotenoids was observed in culture added with 0.005% treatment level at 6th day (**Table 1**). Carotenoid increased at 0.005 and 0.01% treatment till 8th day and decreased thereafter as comparison to control. (**Table 1**).

Table 1. Effect of various doses of Etbr on Carotenoid (in mg /g).

	2ndday	4th day	6th day	8th day	10thday	12th day
Control	247±0.02	.579±0.04	.910±0.06	.982±0.06	.812±0.05	.479±0.04
.005%	457±0.04	.828±0.06	1.646±0.09	1.076±0.06	.636±0.05	.575±0.05
01%	.319±0.03	.414±0.04	1.212±0.08	1.008±0.06	0.554±0.05	0.541±0.05
02%	.373±0.03	.525±0.04	1.102±0.08	0.994±0.06	0.443±0.04	0.446±0.04
04%	.424±0.04	.424±0.04	1.011±0.05	0.887±0.06	0.338±0.03	0.332±0.03
08%	.381±0.03	.387±0.03	.516±0.04	0.810±0.06	0.219±0.02	0.228±0.02

Table 2. Effect of various doses of EtBr on Chlorophyll (in mg /g).

	2ndday	4th day	6th day	8th day	10thday	12th day
Control	1.981±0.57	1.388±0.40	2.627±0.64	3.205±0.98	2.952±0.80	2.144±0.61
.005%	1.413±0.52	1.210±0.29	1.338±0.57	1.838±0.67	2.991±0.82	0.818±0.30
.01%	1.426±0.53	1.314±0.30	1.678±0.61	2.645±0.67	1.331±0.30	0.734±0.30
.02%	1.429±0.54	1.256±0.26	2.331±0.58	2.454±0.54	0.989±0.31	0.334±0.14
.04%	1.338±0.46	1.283±0.28	1.648±0.51	2.212±0.41	0.686±0.28	0.202±0.09
.08%	1.188±0.38	1.178±0.16	1.414±0.50	1.118±0.16	0.712±0.30	0.191±0.08

Table .3. Effect of various doses of EtBr on Protein (in mg /g)

	2nd day	4th day	6th day	8th day	10th day	12th day
Control	228±1.11	250±1.24	276±1.40	254±1.29	248±1.22	225±1.09
.005%	131±0.47	258±1.31	314±2.03	300±1.98	261±1.36	258±1.34
.01%	176±0.58	252±1.26	280±1.86	290±1.96	280±1.48	290±1.52
.02%	154±0.46	256±1.30	414±2.58	402±1.32	381±1.30	381±1.30
.04%	132±0.48	279±1.41	266±1.39	524±3.3	344±1.29	230±1.12
.08%	150±0.45	261±1.36	260±1.35	270±1.85	320±2.00	144±0.43

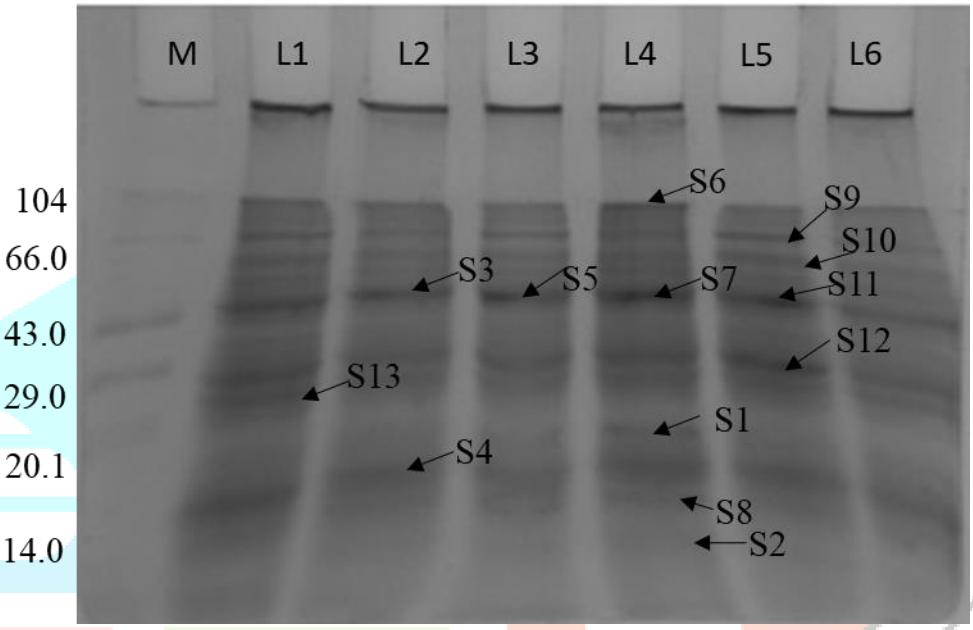

Fig. 1. Coomassie brilliant blue stained polypeptide profile of extracted protein separated by SDS-PAGE. The protein were extracted from *D. salina* under EtBr stress treatment. M: Marker in kDa, L1: control, L2: 0.005%, L3:0.01%, L4:0.02%, L5:0.04%, L6:0.08% of EtBr treated culture.

Overall Maximum chlorophyll was observed in control on 8th day. Chlorophyll was slightly reduced in EtBr addition at all treatment dose when compared to control as increase in time intervals and dose level. (**Table 2)**

Overall maximum protein was observed in control on 6th day. Among all treatment dose maximum protein content was observed in 0.04% treatment dose at 8th day i.e. 524mg/g. Mostly the amount of protein decrease with increase in treatment dose (**Table 3)**

Culture added with EtBr 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 EtBr resolved in SDS PAGE. After staining and de-staining, gel was scanned and photographs were taken. As compared to control, there were two newly expressed polypeptide bands such as S1 and S2 in 0.02% EtBr treated *D. salina* (as shown in L4 of **Fig. 1)** As compared to control, there were some highly expressed polypeptides such as polypeptide bands S3 and S4 in 0.005% EtBr (as shown in L2 of Fig. 1), S5 in 0.01% EtBr (as shown in L3 of Fig. 1), S6, S7 and S8 in 0.02% EtBr, (as shown in L4 of Fig. 1) and S9, S10, S11 and S12 in 0.04% EtBr (as shown in L5of Fig.1). As compared to control with treated culture, there was one polypeptide band S13 in control which was reduced approximately in all treated culture (as shown in L1 of **Fig. 1)**

IV. DISCUSSION

Algae are unique group of living photosynthetic organisms that are present in every type of environment, such as oceans, ponds, rivers, lakes on rocks and on ice etc. Water occupies 2/3 area of earth, and thus the algae appears in largest amount (Khola and Ghazala, 2012). In recent scenarios algae is enormously important in environmental control because it is cheap biomaterial which is utilized in removal of oxides of sulphur and nitrogen, heavy metal, waste water, and 50% fixation of carbon dioxide which are toxic for environment (Ogbonna *et al*., 2000). Various physical factors such as nutrients, light, temperature, pH and salinity effects their biomass and biofuels production (Bartley *et al*. 2016).

Algae can enhance their biomass extremely consuming some nutrients and scarce energy requirements even some species reproduce their cells only within 5 hours (Huber and Dale, 2009). Phycology is the basic and essential tool for the study of molecular structure, biochemistry, cell, ecology, genetics, cell biology etc. of various metabolism of algae.

The phenanthridine dye ethidium bromide (EtBr) is a well-known inhibitor of mitochondrial DNA (mtDNA) replication and transcription (Nass, 1969). Also, EtBr is known to induce mtDNA mutations in *Saccharomyces cerevisiae*, converting the cells to respiration deficient (Slonimski et al., 1968). Even though EtBr is known as an inhibitor and mutagen it is still being used in the molecular biological laboratories as intercalators to visualize DNA (Hayashi and Harada, 2007). Not alone the effective intercalation and efficient fluorescence property of the EtBr makes its use preferable with molecular biologists but most importantly its cost effectiveness, when compared to its alternatives. Recently, EtBr is used in correlation study employing *Mycobacterium smegmatis* as test organism. The study relates EtBr transport across the cell-wall of *Mycobacterium smegmatis* with antibiotic resistance (Rodrigues et al., 2011). Usually, EtBr emits weak fluorescence in aqueous solution (outside cells) and becomes strongly fluorescent when concentrated in the periplasm of gram-negative bacteria and in the cytoplasm of gram-positive bacteria. As long as EtBr is not intercalated between nucleic bases of DNA, it is subject to extrusion. When it is intercalated the binding constant is sufficiently strong to keep EtBr from access to the efflux pump system of the bacterium (Sharples and Brown 1976).

In the present investigation optical density and dry weight all parameters decreased as compared to control. Overall maximum amount of carotenoids was observed in culture added with 0.005% treatment level at 6th day. Carotenoid increased at 0.005 and 0.01% treatment and decreased thereafter as comparison to control. Overall Maximum chlorophyll was observed in control on 8th day. Chlorophyll was slightly reduced in EtBr addition at all treatment dose when compared to control at increase in time intervals and dose level. Overall maximum protein was observed in control on 6th day. Among all treatment dose maximum content was observed in 0.04% treatment dose at 8th day.

According to Sturelid (1971) most of the physiological chromosomal aberrations appear due to the damage caused during replication of DNA by alkylation. The chromosomal bridges with fragments, single bridge, dicentric bridges observed were probably due to the chromosomal stickiness at earlier stages. The production of dicentric bridges was mainly due to the spontaneous breakage of meiotic chromosome (Lewis and John, 1966).

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.

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