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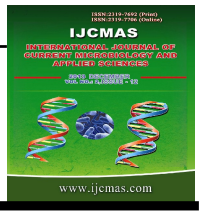

Original Research Article

Generation of pigment mutants of *Chlamydomonas reinhardtii* CC-124 and investigation of the mutants for evaluating the mutability of the waste water ecosystems

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A B S T R A C T

The induced mutagenesis method derived pigment mutants of a green microalga *Chlamydomonas reinhardtii CC-124*, their pigment composition and their ability to assess the mutability of contaminated aquatic ecosystems were studied. In the present study, 14086 (colonies)mutants were obtained from exposure of the wild strain *Chlamydomonas reinhardtii CC-124* to 1, 2, 3, 5 minutes of (UV) irradiation. After screening these (colonies) mutants, revealed four pigmented mutants (*124y-1*, *124p-1*, *124y-2*, *124p-2*). Compared with the wild type *CC-124*, these mutants are characterized by a decrease of chlorophyll a & b content and an increase of carotenoids. The lowest decrease in chlorophyll a was 3-4 folds ,while the highest increase in carotenoids was 2- 4 folds . The result of biotest, using the resulting pigment mutant of *Chlamydomonas reinhardtii124y-1*, showed that the mutagenic activity was observed significantly in both water of the Tekeli river and the Pavlodar oil refinery in Kazakhstan and the waste water of the Pavlodar oil refinery was of high-toxicity degree while the water of the Tekeli river was of medium-toxicity degree.

Keywords

UV mutagenesis; *Chlamydomonas reinhardtii;* Biotesting.

Introduction

One of the most and serious ecological problems is mutagenic pollution of the natural environment. Therefore, detection of mutagenic compounds in samples taken from natural habitats is of special interest. The problem of the presence of mutagenic chemicals in natural habitats is very important because such compounds are capable of inducing serious diseases, including cancer and elicit deleterious effects on living organisms (Shigaeva *et al*.,1994) as well as are expensive and time consuming (Wegrzyn and Czyz, 2003). Biological assays may be an alternative to chemical analysis when detecting the presence of mutagenic compounds in the environment .Although no currently available biological test can provide

detailed and precise information about a possibility to answer the question whether examined samples contain mutagens at levels potentially dangerous for organisms. Therefore, it seems that the most reasonable strategy for testing environment samples is to use a biological assay as a preliminary test to detect the presence of mutagenic compounds. Biotesting is one of the biological methods based on native or genetically modified microorganisms as test -species have already successfully been applied to environmental toxicity, genotoxicity assessment as well as it depends on the easy accessibility and/or maintenance of the organisms in the laboratory Nendza, (2002); Allan, *et al*., (2006). Soil unicellular green alga *Chlamydomonas reinhardtii* Dang. is a superb model organism for study of a wide range of biological questions in areas such as flagellar function, photobiology and photosynthesis research Stolbov, (1995) , Pedersen *et al*., (2006) Schmidt *et al*,(2006) because of its clear genetic back-ground.

C. reinhardtii is a unique biological material that contains three genetic systems located in the nucleus, chloroplast, and mitochondria (Merchant *et al*., 2007). In addition, it has rapid growth, a short breeding cycle, and low-cost cultivation. The study of the consequences of the action of mutagenic substances on wild and mutant strains of interest is not only in terms of expanding our knowledge of the biological effects of factors that pollute the ecosystem, but also the emergence of opportunities receipt of test systems for genetic monitoring of the environment. Our goal in the current study is obtaining pigment mutants of green microalga *ChlamydomonasreinhardtiiCC-124* by induced mutagenesis and tested to evaluate their effect of the mutability of contaminated aquatic ecosystems.

Materials and Methods

Microalgal strain and cultivation conditions

The green soil alga *Chlamydomonas reinhardtii CC-124* obtained from Kazakhstan National university- Al-Farabi, Biotechnology Department culture collection. Microalga was cultured and grown in1000 ml conical flasks containing L2-minimal (L2m)media (Harris,1998).Algae were cultured at 25±0.5 ◦C with a fluorescent light intensity of approximately(6 W/m^2). Cells in the exponential growth phase were used and the initial cell density was about 1X $x10^6$ cells / ml. The number of cells was determined by counting using Goryaev'shemocytometer under a light microscope

UV irradiation mutagenesis of *Chlamydomonas reinhardtii* CC-124

According to the description of Harris (1998), microalgal cells of 4 mL in a logarithmic phase were placed in a 9 cm Petri dish, forming a thin layer covering the bottom. The dish was exposed to a UV-A lamp ($5W/m^2$) for 1, 2 3, 5, min, respectively. After UV irradiation, the irradiated and un-irradiated (control) cells taken from different dilutions, were spread immediately on respective agar plates with L2m media and were kept in the dark for 24 hour to prevent photoreactivation, and then grown for 15 days after dividing the dishes into two groups ,the first one were kept in the dark(under heterotrophic condition) and the second one grown under constant light(under phototrophic condition).The identification of the

mutants was carried out immediately after exposure and after daily dark repair of cells to prevent the increase of frequency of various kinds of mutations due to errors in DNA replication.

Growth curve and the percentage abundance of survivors

The ratio of cell survival was assessed by determination of the percentage of the surviving macro colonies after irradiation exposure dose corresponding to that of the unexposed colonies of the same dilution**.** Survival curve was constructed by plotting the log of the surviving fraction against the time exposure

Subculturing of the resulting mutant cells in a liquid media to get survival subclones maintaining phenotypic characters

Approximately , 14086 morphological surviving subclones had been formed after UV exposure. Out of this number of colonies, 12 mutant subclones were selected for further breeding to study size and shape.L2m was used as a growing medium on the selection of mutant subclones of *Chlamydomonas reinhardtii*. Subclones were screened for maintaining phenotypic characters throughout series of passages .There have been up to ten consecutive rounds of selections.

Analysis of pigment composition of the selected 4 mutanized subclons

Spectrophotometry method was used according to Merchant *et al*., (2007). The calculation of the concentration of the pigments was determined by the optical density of pigment solutions at appropriate wavelength.

UV irradiation mutagenesis of the selected 4 subclons

(124y-1, 124p-1, 124y-2 and 124p-2 mutants) resulted in 3 new colonies characterized by different green colors pigments (dark green ,light green and yellow green) to select the best one as a test organism.

Method of determining the mutagenicity of water samples by introducing a test organism in the experimental and control samples, with subsequent incubation and determination of the frequency occurrence of reverse mutations.

To identify substances that have the genetic activity on cells, pigment mutants kept in the test water, and calculate the incidence of forward and reverse mutations (counting the number of revert ants). Chlorophyll b-deficient mutants were selected among the light-stable revert ants by the level of fluorescence. The fluorescence level is mainly determined by chloroplasts antenna of chlorophyll a PSII. The excitation energy of PSII is a light-harvesting Chl a / b-protein complex that contains 80% of the total chl b. In this regard, the absence of chl b reduces fluorescence of the cells. The fluorescence of chl b excited wide bands of light at 469-640 nm. Chl florescence in the cells was observed through KS-2 filter. The absorption spectra of aqueous suspensions of cells were recorded with spectrophotometers SF-10 and SF-18. The ratio of chl a/chl b were determined by the fluorescence method.

Selection the pigmented mutant that can be used as a test organism

Depending on UV irradiation as a mutagenic agent we considered the percentage of revert ants mutants that

were induced by UV irradiation as control and could be used under comparable with the percentage of others revert ants due to contaminated water . The pigmented mutant of *Chlamydomonas reinhardtii 124y-1* was selected in terms, of more stability, its chl b not detected and more carotene content than the others .The maximum frequency of revertants were detected after 3 mins .

Method of determining the mutagenicity of water samples by introducing a test organism in the experimental and control samples, with subsequent incubation

The test organism was grown in a media added with the selected water sample under testing for the mutability. The assessment of water mutability was carried out by counting the number of cells revert ants.

Results and Discussion

A mutagen is anything that changes the genetic material of an organism. Ultraviolet (UV) irradiation has a strong mutagenic agent, compared with chemical mutagenesis, UV mutagenesis offers many advantages such as less pollution, simple operation, and sterile cultivation condition (Huang *et al*., 1993) Several successful cases on microalgae strains for UV mutagenesis have been documented (Zhang *et al*., 2009; Danil'chenko *et al*., 2002Deng *et al*.,2011).In the current study ,UV mutagene can induce the frequency of mutation in *Chlamydomonas reinhardtii* CC-124 .After 1 min. exposure, the number of survival cells represents 31% and the grown colonies did not differ from the control group in terms of they had a medium size and a green color. Upon irradiation of the organism for 2 min., a significant reduction in the number of viable cells reached to 10.5% , in addition to a heterogeneity of colonies i.e (large, medium sizes and very fine, green, light green and a dark green color). The number of grown cells after 3 min. exposure of irradiation was 4.5%.and the grown colonies were characterized by different sizes and dark green color. At 5-min exposure, significantly no algal growth , was observed. It is clear that UV light has a lethal effect on the cells viability and created opportunities for optimal formation of morphological mutations due to its ability to induce highly efficiently DNA damage with a survival curve being C-shape (Fig.1&2) this is in agreement with the reports of many researchers on the effect of UV light on algal microorganisms (Cadet *et al*., 1992; Danilchenko, *et al* ., 2002; Wu*et al*.,2005; Deng*et al*.,2011; IKehata and Ono,2011)

Exposure of *Chlamydomonas reinhardtiiCC-124* to UV radiation with $5W/m^2$ for 1-5 minutes out of 130 000 cells of *Chlamydomonas reinhardtii s*train, 14086 morphological surviving sub clones had been formed. As a result, mass selection, without verification of the genotype in the various culture conditions we obtained subclones, which are characterized by changing the size and color of the colony. These subclones will be divided into six groups.

Under photoautotrophic culture conditions:

Group 1 - subclones green color and large size (A) - 18%;

Group 2 - subclones green color and microscopic size (B) - 32%;

Group 3 - subclones light green color and medium-sized (C) - 33%;

Group 4 - subclones yellow color and medium size (D) -17%.

The control group is consisted of colonies of green color and medium size.
Under heterotrophic culture conditions:
Group 5 - subclones light green color and medium size (E) - 68%;
Group 6 - subclones yellow color and medium sized (F) - 32%.
The control group is consisted of colonies of green color and medium size

Analysis of the output of various mutant sub clones under photoautotrophic showed that the highest percentage of subclones (33%) of the total subclones are green color and medium – sized.Under heterotrophic condition the highest percentage of subclones (68%) are light green color and medium sized. (Table1).For further investigation of the 12 colonies by repeated breeding. Subcolonies were selected from 4 groups (3,4,5,6)which have preserved the characters(yellow and light green color). They are nominated as 124y-1 and 124p-1, obtained under photoautrophic conditions, and 124y-2 and 124p-2 ,obtained under hetrotrophic conditions. Extraction of the mutant pigments was carried out on the fifth day of growth medium cultures with sodium acetate in the light showed that(Table 2) ,a decrease in the content of chl a, and chl b not detected, wheras there was an increase of carotenoids compared with that the wild strain.The carotenoid contentin the cells of C*hlamydomonas reinhardtii* pigment mutants *124y-1, 124p-1, 124y-2* was 15.35, 12.19 and 23.36 µg/ml, respectively compared with wild strain (8.12 µg/ml)i.e the increase by 2-4 times.

Generally under optimal light conditions, there is a certain balance between the pigment content in the algal cells which is a charasterstic feature of the species.Under exposure to mutagenic agent, the balance would exchange in either direction. UV irradiation can excite the electron shells, resulting in formation of phot-electrons cuasing a variety of chemical reactions leading to mutations. Upon irradiation ,the cells begin to synthsise carotenoids and quantity of carotenoids produced depends on the intensity of UV radiation.Concerning of UV effect on the photosynthetic pigments of plants and algae, some studies (Solovchenkand Merzlyak, 2008) revealed that the synthesis of pigments is blocked, retardation of cell growth as well as there is a strong trend towards increased levels of carotenoid in pigments of mutants. In confirming of our data(Demmig-Adam1998)reported that in response to excess of light ,a rapid increas in carotenoids propably reflecting the permanently increased needs for photoprotection. Also Kleinegris *et al*.,(2010) stated that *Dunaliella salina* algae is bombarded with the full brunt of solar UV (ultraviolet) radiation and has evolved a novel mechanism for defending itself from its damaging effects. More than 8% of its dry body mass is β-carotene, more than any other organism that produces the compound.In spite of some literatures reported that response of carotenoids to UV is variable:decreased carotenoids level were observed under UV (Kirchgebner *et al*., 2003) but they were also stimulated by UV (Xiong and Day,2001). The decrease of chl a and b under elevated UV has also been reported by (Bidigare *et al*., (1993); Hagen *et al*., (1993); Deckmyn*et al*.,1994; Remias *et al*., 2010). Regarding the selection of test organism for determination of the mutagenicity of water samples, the selected mutant pigment 124y-1,124p-1 , 124y-2 and 124p-2 exposed to UV irradiation and the resulting 3 new types characterized by discoloration of the colonies (dark green,light green and faint green color).

Table.1 Frequency of mutations in wild-type cells*CC-124 Chlamydomonas reinhardtii* at various doses

Exposure time (minutes)	**Total number of colonies**	**Cell viability**	**The number of normal colonies**	**The number of mutant colonies**	**Identification of selected colonies**
0	90 000	92-100%	126000	-	-
1	39060	31%	31248	7812	A
					B
2	13230	10.5%	8997	4233	A
					B
					C
					D
					E
					F
3	5670	4.5%	3629	2041	E
					F
5	-	-	-	-	-

Table.2 The content of chlorophyll and carotenoid pigment mutants in *Chlamydomonas reinhardtii*

Cipher of the strain	**chlorophyll *a* content µg/10^6 cells**	**Chlorophyll*b*** content **µg/ 10^6 cells**	**Carotenoid content µg/ 10^6 cells**
CC-124	28.73±5.72	13.77±2.42	8.12 ± 2.42
124y-1	5.65±2.35	-	15.35±2.65
124p-1	6.32±2.38	4.56±3.63	12.19±1.48
124y-2	-	-	23.36±2.25
124p-2	6.65±3.21	-	8.69±2.30

Table.3 The study of the action of UV light on the pigment mutants of green microalga *Chlamydomonas reinhardtii*

The incidence of	**control**	**UV Light (**Minutes)	
		1	**3**
124y-1	$5.5x10^{-4}$	$4.5x10^{-4}$	$4.2x10^{-3}$
124y-2	$5.5x10^{-4}$	$4.1x10^{-4}$	$3.8x10^{-3}$
124p-1	$5.5x10^{-4}$	$3.9x10^{-4}$	$3.6x10^{-3}$
124p-2	$5.5x10^{-4}$	$3.8x10^{-4}$	$3.2x10^{-3}$
Total number of revertants color change	$2.5x10^{-5}$	$7.2x10^{-6}$	$3.2x10^{-5}$

Table.4 The study of genetic activity of various wastewater samples of Pavlodar refinery

Incidence	Control	Experiment(sample №)		
		1	2	3
Pigment mutants	$< 6.5x10^{-4}$	$9.1x10^{-4}$	$0.5x10^{-3}$	$2.6x10^{-3}$
Revertants color change	$<10^{-5}$	$0.8x10^{-4}$	$2.4x10^{-4}$	$6.3x10^{-4}$

Figure.1 Effect of UV irradiation on survival of wild-type cells of *Chlamydomonas reinhardtii CC-124*

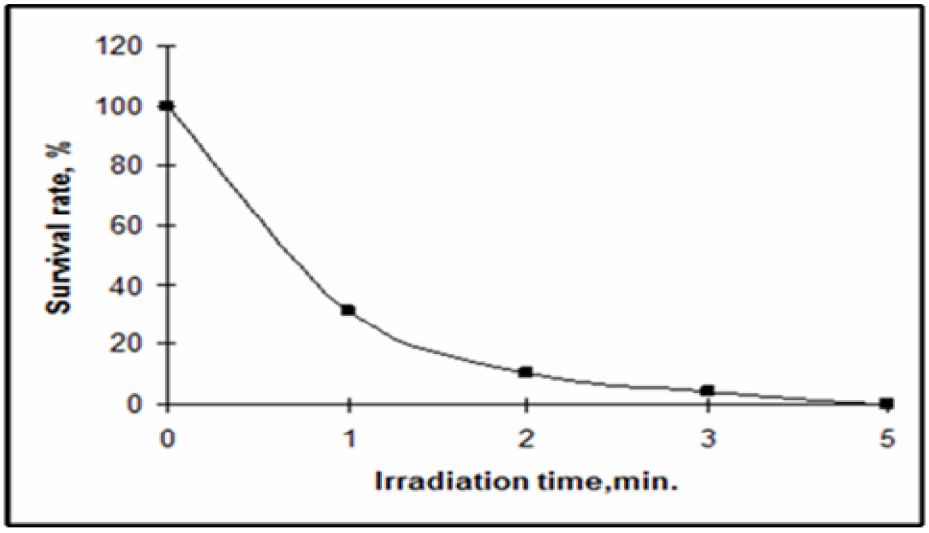
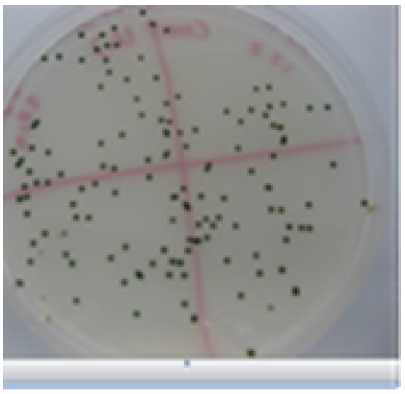
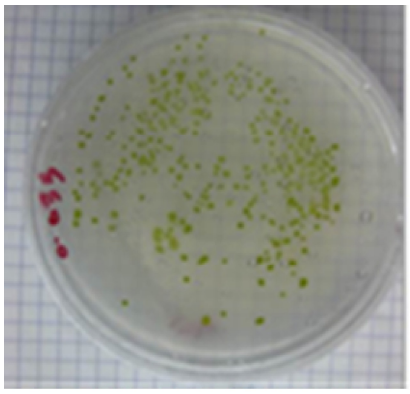


Fig.2 a) Colonies of normal wild type with dark green colour b) Mutant colonies with light green colour

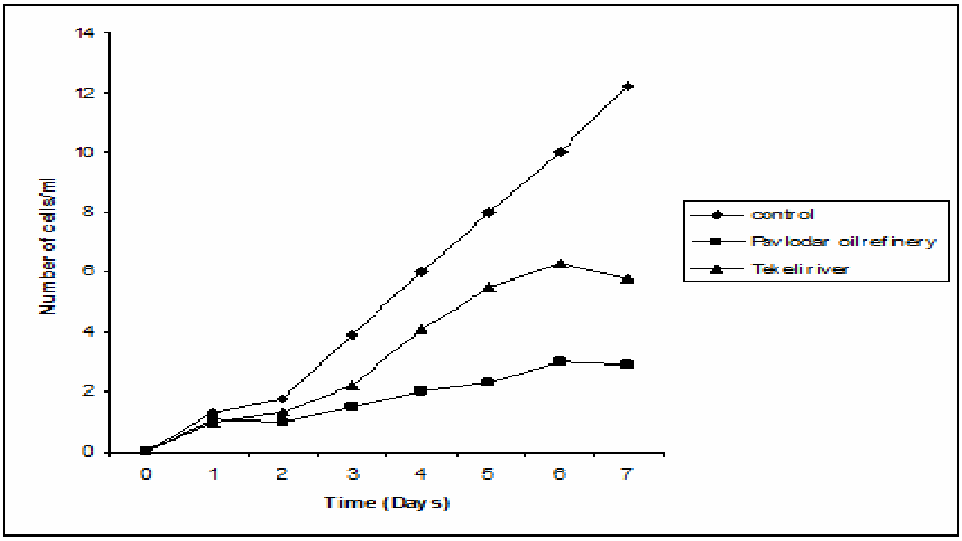


A



B

Fig.3 Effect of mutagenic activity of different types of polluted waters on Survival pigmented mutant *124y-1*.



The maximum frequency of mutations were observed after 3 min.of UV irradiation. At the same time there was a significant increase in the incidence of direct mutation of pigment (Table 3). Among the 3 mutants,we selected 124y-1 mutant for biotesting since, it has more stability , an increse in carotenoid and chl b not detected.This result in alignment with that of (Parasad *et al*., 1993) that the sensivity of photosynthetic pigment to UV was in order of :chl b>chl a>carotenoid To assess the mutagenicity of water samples from Tekeli and Pavlodar oil refinery in Kazakhstan the selected test organism was undersubsequent incubation in the experimental and control samples to determine the occurrence frequency of reverse mutations .If the tested sample contains promutagens mutagenic chemical compound ,they will induce a reverse mutation restoration of wild –type phenotype .Consequently Samples of Tekeli river effluent were toxic and cuased an inhibition of cell growth of the mutant 124y-1. As shown in (Fig.3) the cells of the test organism were 1.5 times less than the control compared with the first days of the experiment.Its mutagenicactivity against Chlamydomonas reinhardtii strain 124y-1 was observed, as evidenced by the lack of forward and reverse mutations.Also Samples of Wastewater of Pavlodar refinery were toxic and have mutagenic activity, as induced by the appearance of the direct and reverse mutations was evident, as shown by a slight increase in the incidence of light-stable revertants (Table 4).

In the present study ,wastewater samplesfrom Tekeli river and Pavlodar oil refinery in Kazakhstan, were evaluated for theirecotoxicological effects using 124y-1 mutant.The water of Tekeli river was of medium toxicity and wastewater of Pavlodar refinery was of high toxicity. The current study may be allowed us to use in the future, UV radiation (radiation dose was 3minutes) as a positive control to determine the toxicity of toxicants from contaminated ecosystems.In our opinion, the system of assessment of water quality based on microalgae is a promising and can be further improved by the

development of new testing methods, as well as expanding the range of use of mutants.

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