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# Generation and characterization of pigment mutants of *Chlamydomonas reinhardtii* CC-124

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**The induced mutagenesis method for deriving pigment mutants of a green microalga, *Chlamydomonas reinhardtii CC-124* and their pigment composition as well as ability to assess mutability of contaminated aquatic ecosystems were studied. In the present study, 14086 mutants (colonies) were obtained by exposure of the wild strain, *C. reinhardtii CC-124,* to 1, 2, 3, 5 min of ultraviolet (UV) irradiation. After screening, these mutants (colonies) revealed four pigmented mutants (*124y-1*, *124p-1*, *124y-2* and *124p-2*). Compared to the wild *CC-124*, these mutants are characterized by a decrease in chlorophyll a & b content and an increase in carotenoids. The lowest decrease in chlorophyll a was 3 to 4 folds, while the highest increase in carotenoids was 2 to 4 folds. The result of bio-test, using the resulting pigment mutant of *C. reinhardtii 124y-1* showed that mutagenic activity was observed significantly in both Tekeli River and Pavlodar Oil Refinery in Kazakhstan; the waste water of the Pavlodar Oil Refinery had high-toxicity while the water of the Tekeli River had medium-toxicity.**

**Key words:** Ultraviolet (UV) mutagenesis, *Chlamydomonas reinhardtii*, biotesting.

## INTRODUCTION

One of the most 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); they are also expensive and time consuming (Wegrzyn and Czyz, 2003).

Biological assays may be an alternative to chemical analysis when mutagenic compounds are detected in the environment. Although, no currently available biological test can provide detailed and precise information on whether examined samples contain mutagens at the levels that are potentially dangerous for organisms. Therefore, it seems that the most reasonable strategy for testing environmental samples is to use a biological assay as a preliminary test to detect the presence of mutagenic compounds. Bio-testing is one of the biological methods based on native or genetically modified micro-organisms as test species have already been applied successfully to environmental toxicity, genotoxicity assessment. It depends on the easy accessibility to 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 the 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

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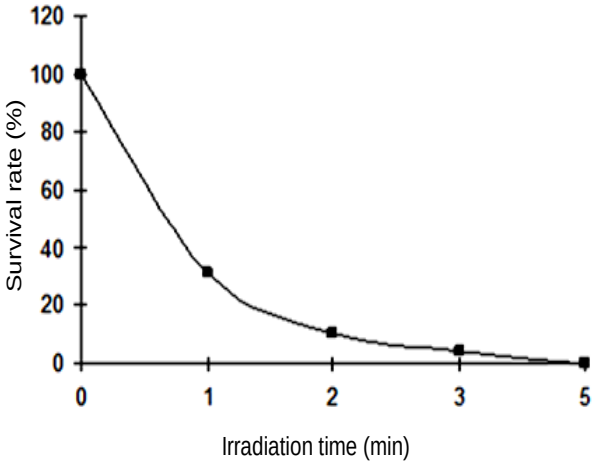


**Figure 1.** Effect of UV irradiation on survival of wild-type cells of *Chlamydomonas reinhardtii CC-124.*

(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 to obtain pigment mutants of green microalga, *C. reinhardtii CC-124* by induced mutagenesis and to evaluate the effect of the mutability of contaminated aquatic ecosystems.

## MATERIALS AND METHODS

### Microalgal strain and cultivation conditions

The green soil alga, *C. reinhardtii CC-124* was obtained from Kazakhstan National University, Al-Farabi, Biotechnology Department culture collection. Microalga was cultured and grown in 1000 ml conical flasks containing L2-minimal (L2m) media (Harris, 1998). It was 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 ×10$^6$ cells / ml. The number of cells was determined by counting, using Goryaev's hemocytometer 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 and 5 min, respectively. After ultraviolet (UV) irradiation, the irradiated and un-irradiated (control) cells taken from different dilutions were spread immediately on respective agar plates with L2m media; they were kept in the dark for 24 h to prevent photoreactivation, and then grown for 15 days after dividing the dishes into two groups. The first one was kept in the dark (under heterotrophic condition) and the second one was 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 determining the percentage of the surviving macro colonies after irradiation exposure dose corresponded 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 of exposure (Figure 1).

### Sub-culturing of the resulting mutant cells in a liquid media to get survival sub-clones maintaining phenotypic characters

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

### Analysis of pigment composition of the selected 4 mutanized sub-clones

Spectrophotometry method was used according to the study of 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 sub-clones (*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 the substances that have genetic activity on cells, pigment mutants were kept in the test water and the incidence of forward and reverse mutations was calculated (counting the number of revertants). Chlorophyll b-deficient mutants were selected among the light-stable revertants 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 to 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 ratios of chl a/chl b were determined by the fluorescence method.

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

Depending on UV irradiation as a mutagenic agent, we considered the percentage of revertants mutants that were induced by UV

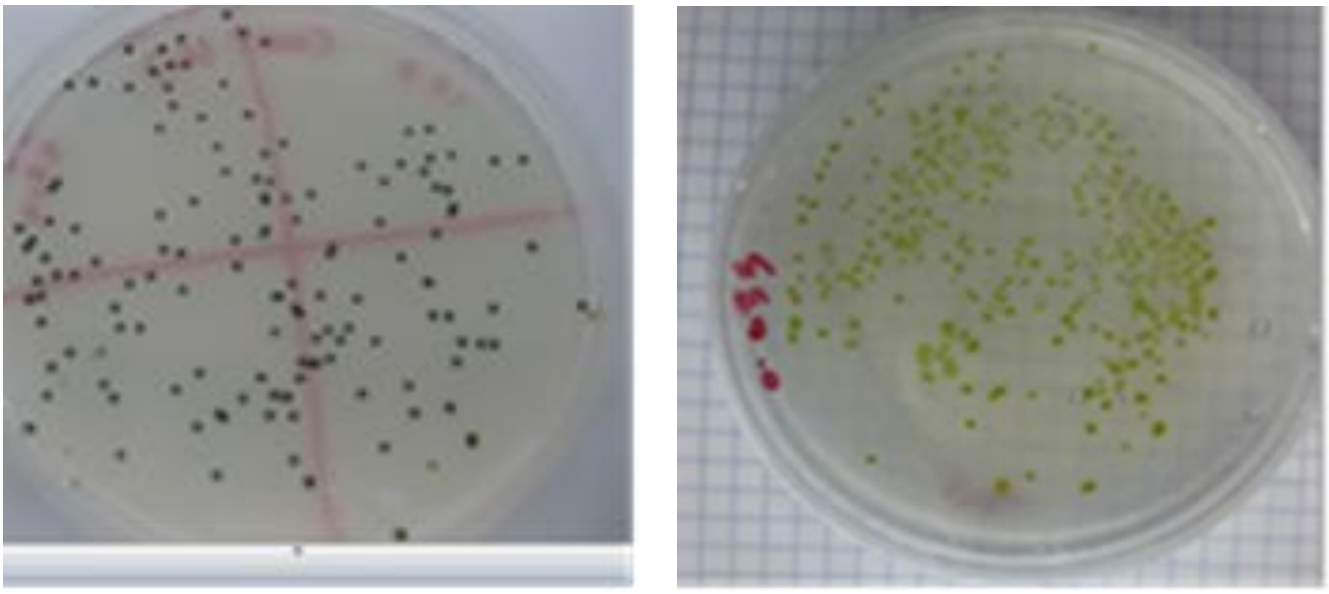


**Figure 2.** Colonies of normal wild type with dark green colour (a), mutant colonies with light green colour (b).

irradiation as control and could be comparable with the percentage of other revertants due to contaminated water. The pigmented mutant of *C*. *reinhardtii 124y-1* was selected because it is more stable its chl b is not detected and has more carotene content than the others. The maximum frequency of revertants was detected after 3 min.

**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 mutability. The assessment of water mutability was carried out by counting the number of cells revertants.

## RESULTS AND DISCUSSION

A mutagen is anything that changes the genetic material of an organism. The most famous one is UV irradiation. Ultraviolet (UV) irradiation has a strong mutagenic agent, compared to chemical mutagenesis. UV mutagenesis offers many advantages such as less pollution, simple operation and sterile cultivation condition (Huang et al., 1993). Several successful cases of microalgae strains by using UV mutagenesis have been documented (Zhang et al., 2009; Danil'chenko et al., 2002; Deng et al., 2011). In the current study, UV mutagenesis can induce the frequency of mutation in *C*. *reinhardtii* CC-124. After 1 min exposure, the number of survival cells was 31% and the grown colonies did not differ from the control group in terms of their medium size and green color. Upon irradiation of the organism for 2 min, a significant reduction in the number of viable cells reached 10.5%, in addition to a heterogeneity of colonies (large, medium sizes and very fine, green, light green and dark green color). The number of grown cells after 3 min exposure to 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 efficient DNA damage with a survival curve of C-shape (Figures 1 and 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).

In exposing *C*. *reinhardtii CC-124* to UV radiation with 5 W/m$^2$ for 1 to 5 min, out of 130 000 cells of *C*. *reinhardtii s*train, 14086 morphological surviving sub clones were formed. As a result, in the mass selection without verification of the genotype in the various culture conditions, we obtained sub clones, which are characterized by changing size and color. These sub-clones are divided into six groups.

Under photoautotrophic culture conditions:

Group 1: Green color and large size (A) - 18%;
Group 2: Green color and microscopic size (B) - 32%;
Group 3: Light green color and medium-sized (C) - 33%;
Group 4: Yellow color and medium size (D) -17%.

The control group consists of colonies of green color and medium size.

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

| Exposure time (min) | 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<br>B |
| 2 | 13230 | 10.5 | 8997 | 4233 | A<br>B<br>C<br>D<br>E<br>F |
| 3 | 5670 | 4.5 | 3629 | 2041 | E<br>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 |

Under heterotrophic culture conditions:

Group 5: Light green color and medium size (E) - 68%;
Group 6: Yellow color and medium size (F) - 32%.

The control group consists 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 (Table 1). For further investigation of the 12 colonies by repeated breeding, subcolonies were selected from 4 groups (3, 4, 5 and 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; it showed (Table 2) a decrease in the content of chl a and chl b was not detected; whereas there was an increase of carotenoids compared to that of the wild strain. The carotenoid content in the cells of C. *reinhardtii* pigment mutants *124y-1*, *124p-1*, *124y-2* was 15.35, 12.19 and 23.36 µg/ml, respectively compared to the wild strain (8.12 µg/ml); that is, an increase by 2 to 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 photo-electrons; this can cause 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 UV effect on the photosynthetic pigments of plants and algae, some studies (Solovchenk and Merzlyak, 2008) revealed that the synthesis of pigments is blocked, there is retardation of cell growth as well as there is a strong trend towards increased levels of carotenoid in pigments of mutants. In confirming our data, Demmig-Adam et al., (1996) reported that in response to excess of light, a rapid increase in carotenoids propably

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

| The incidence | Control | UV light (min) | |
|---|---|---|---|
| | | 1 | 3 |
| 124y-1 | $5.5\times10^{-4}$ | $4.5\times10^{-4}$ | $4.2\times10^{-3}$ |
| 124y-2 | $5.5\times10^{-4}$ | $4.1\times10^{-4}$ | $3.8\times10^{-3}$ |
| 124p-1 | $5.5\times10^{-4}$ | $3.9\times10^{-4}$ | $3.6\times10^{-3}$ |
| 124p-2 | $5.5\times10^{-4}$ | $3.8\times10^{-4}$ | $3.2\times10^{-3}$ |
| Total number of revertants color change | $2.5\times10^{-5}$ | $7.2\times10^{-6}$ | $3.2\times10^{-5}$ |

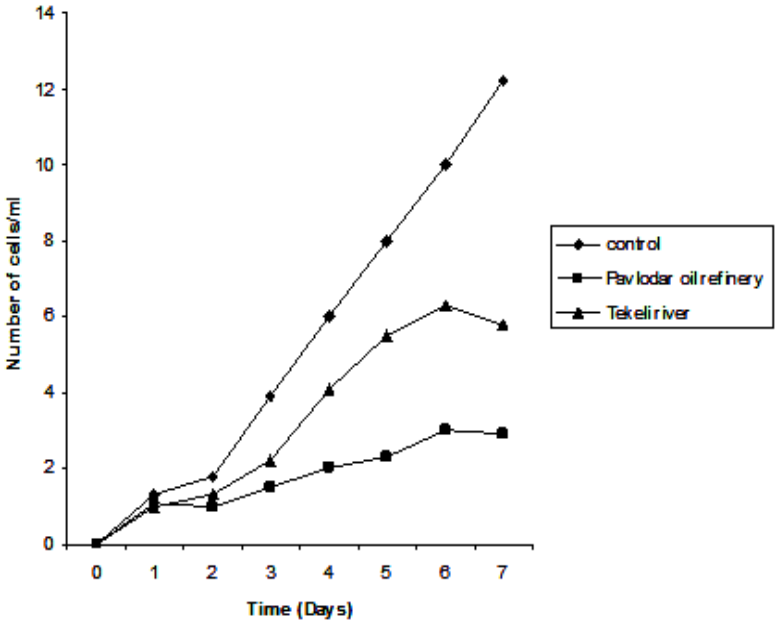


**Figure 3.** Effect of mutagenic activity of different types of polluted waters on survival pigmented mutant *124y-1.*

reflects the permanently increased needs for photoprotection. Also, Kleinegris et al. (2010) stated that *Dunaliella salina* alga 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 reporting that response of carotenoids to UV varies, 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) and Remias et al. (2010). Regarding the selection of test organism for the determination of the mutagenicity of water samples, the selected mutant pigments*, 124y-1*, *124p-1*, *124y-2* and *124p-2* were exposed to UV irradiation and the resulting 3 new types were characterized by discoloration of the colonies (dark green, light green and faint green color). The maximum frequency of mutations was 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 is more stable, has increased carotenoid and lacks chl b. This result is 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 under subsequent incubation in the experimental and control samples to determine the occurrence frequency of reverse mutations. If the tested samples contain promutagens mutagenic chemical compound, they will induce a reverse mutation restoration of wild -type phenotype.

Consequently, samples of Tekeli River effluent were

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

| The incidence | Control | Experiment (sample no.) | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| Pigment mutants | $<6.5\times10^{-4}$ | $9.1\times10^{-4}$ | $0.5\times10^{-3}$ | $2.6\times10^{-3}$ |
| Revertants color change | $<10^{-5}$ | $0.8\times10^{-4}$ | $2.4\times10^{-4}$ | $6.3\times10^{-4}$ |

toxic and cuased an inhibition of cell growth of the mutant 124y-1. As shown in Figure 3, the cells of the test organism were 1.5 times less than the control in the first days of the experiment. Its mutagenic activity against *C. 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 had mutagenic activity, induced by the appearance of the direct and reverse mutations, and shown by a slight increase in the incidence of light-stable revertants (Table 4). In the present study, wastewater samples from Tekeli River and Pavlodar Oil Refinery in Kazakhstan were evaluated for their ecotoxicological 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 allow us to use UV radiation (radiation dose was 3 min) as a positive control to determine the toxicity of toxicants from contaminated ecosystems in the future. In our opinion, the system of assessment of water quality based on microalgae is 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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