Chlamydomonas reinhardtii Exposure to Phenol and Genetic Response

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Abstract

The demonstration that cell count and optical density measurements of *Chlamydomonas reinhardtii* exhibit a linear correlation over the log phase of the algae's growth cycle provides an efficient method of recording *C. reinhardtii*'s growth over time. The exposure of *C. reinhardtii* to a stress, such as phenol, may induce promoters in the algae's genome and alter its genetic expression. Microarray analysis will be used to determine *C. reinhardtii*'s response to phenol on the genomic level. Information from the microarray analysis can be used to genetically engineer an algae biosensor to detect phenol exposure.

1. Introduction

Phenol (C_6H_6O) is a chemical produced through both natural and synthetic processes. As a natural substance, it is found in the air and water. As a commercial product, phenol is present in several consumer goods, such as ear and nose drops and antiseptic lotions. It is also commonly used in many manufacturing processes. Inappropriate disposal can result in an elevated presence of the chemical in the environment. Human exposure to excessive levels of phenol could occur by drinking contaminated surface or ground water or coming into contact with contaminated water near these manufacturing sites. This risk is particularly high for people living near landfills, waste disposal sites, or phenol spills.

According to the Agency for Toxic Substances and Disease Registry [1], the total amount of phenol released into the water was estimated at 72,550 pounds in 1996 [1]. Phenol has been measured in ambient water (from 1.5 to >100 ppb), groundwater (from 1.9 to >10ppb), effluents (up to 53 ppm), and drinking water (not quantified) [1]. The highest levels of phenol have been found in water supplies and discharge waters for industrial activity. For instance, phenol was found in the untreated effluent from a coal conversion plant at 4,780 ppm [1].

When phenol enters the environment, it can remain in water supplies for 9 or more days, but large or repeated introduction of the chemical can remain for greater periods of time. While natural exposure to phenol is not generally harmful, overexposure to the chemical can have serious health consequences. Low level exposure to phenol in drinking water is associated with mouth sores and diarrhea in humans [1]. In addition, experimental exposure of high levels of phenol in drinking water resulted in muscle tremors and loss of coordination in animals [1].

Early and continuous phenol detection could prevent many of these harmful health problems. Current phenol detection methods are laborious, time-consuming, and expensive. Biosensors may be a solution to overcome these difficulties. Biosensors are organisms that incorporate an environmental material and produce a signal that indicates exposure to the stimulus. An algae biosensor for phenol would be useful for detecting its presence in water because of algae's growth in aquatic environments. Samples from a suspected contaminated water source could be exposed to the biosensor in vitro to confirm phenol presence.

The unicellular green alga *Chlamydomonas reinhardtii* is used as a model organism to study genetic response to phenol. The organism is an excellent model because a great deal is known about its genome which has been sequenced. It can grow on liquid and solid mediums

and in both light and dark conditions [2]. Cell growth is also rapid with a doubling time of 5-6 hours when grown under bright light [3].

In this study, growth analysis trials of *C. reinhardtii* grown on phenol were conducted in order to determine the algae's tolerance threshold level to the chemical. Microarray analysis will be used in order to investigate *C. reinhardtii*'s specific transcriptional response to phenol. This technology enables the transcription level of every gene in a cell to be measured. Promoters that are induced by phenol exposure may be revealed. These promoters can be fused with marker genes that can be used as a biosensor for detecting phenol. In addition, the use of optical density (O.D.) as a reliable measure of *C. reinhardtii* growth over time is assessed.

2. Materials and Methods

2.1 *Chlamydomonas* growth curve culture conditions

C. reinhardtii cultures were maintained on Tris-acetate-phosphate (TAP) agar medium at 25°C under continuous light. A single colony of *C. reinhardtii* cells was used to make a starter culture grown in liquid TAP medium. The starter culture was grown under 14 hours of light (150μE) and 10 hours of dark per day at 27°C on a rotary shaker (200 rpm). Four new 50 ml cultures were started in 125 ml Erlenmeyer flasks with 100 μl starter culture and liquid TAP medium. Cultures were returned to the previous growing conditions. Cells were counted daily over 7 days with a hemacytometer. The O.D. of the cultures was also measured using a 200 μl culture aliquot and a microplate reader at 750 nm.

2.2 *Chlamydomonas* phenol growth analysis culture conditions

A stock solution of 0.5M phenol solution was prepared by dissolving crystalline phenol in deionized water. *C. reinhardtii* cultures were maintained on Tris-acetate-phosphate (TAP) agar medium at 25°C under continuous light. A single colony of *C. reinhardtii* cells was used to make a starter culture grown in liquid TAP medium. The starter culture was grown under 14 hours of light (150μE) and 10 hours of dark per day at 27°C on a rotary shaker (200 rpm). Fresh 50 ml cultures were started in 125 ml Erlenmeyer flasks with 1 ml starter culture, TAP, and phenol (0, 2.5, 5.0, 7.5, 10 mM phenol). Three replicates of each phenol/TAP concentration were prepared. Cultures were returned to the previous growing conditions. The O.D. of the cultures was measured daily with a microplate reader at 750 nm over 3 days once the cultures had reached a minimum absorbance of 0.05.

3. Results

3.1 *Chlamydomonas* growth curve conditions

Growth curve studies were conducted in order to determine the reliability of O.D. as a measure of *C. reinhardtii* growth over time. Plots of both cell count and O.D. versus time produced characteristic, sigmoidal growth curves (Figures 1 and 2). Optical density was plotted versus cell count (Figure 3) to illustrate the linear correlation between the two measurements

over the log phase of *C. reinhardtii*'s growth cycle. A linear correlation ($r^2 = 0.9971$) was found between approximately 0.05 and 0.5 absorbance (Figure 3).

Figure 1

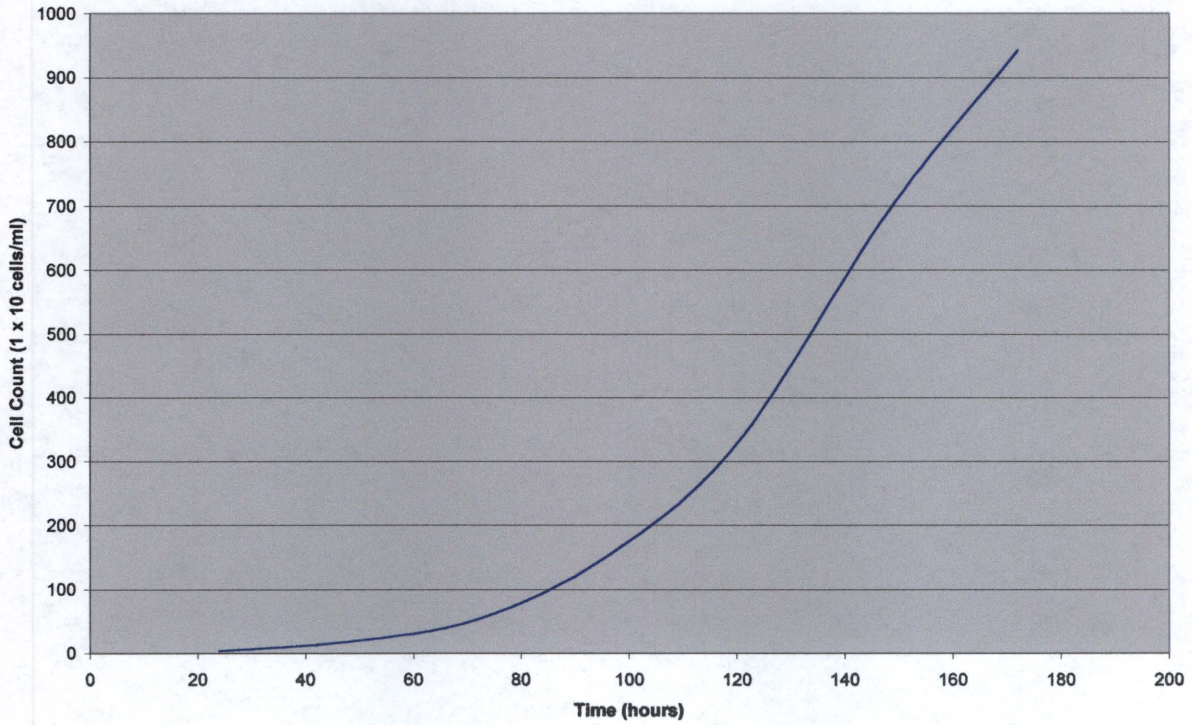


Figure 2

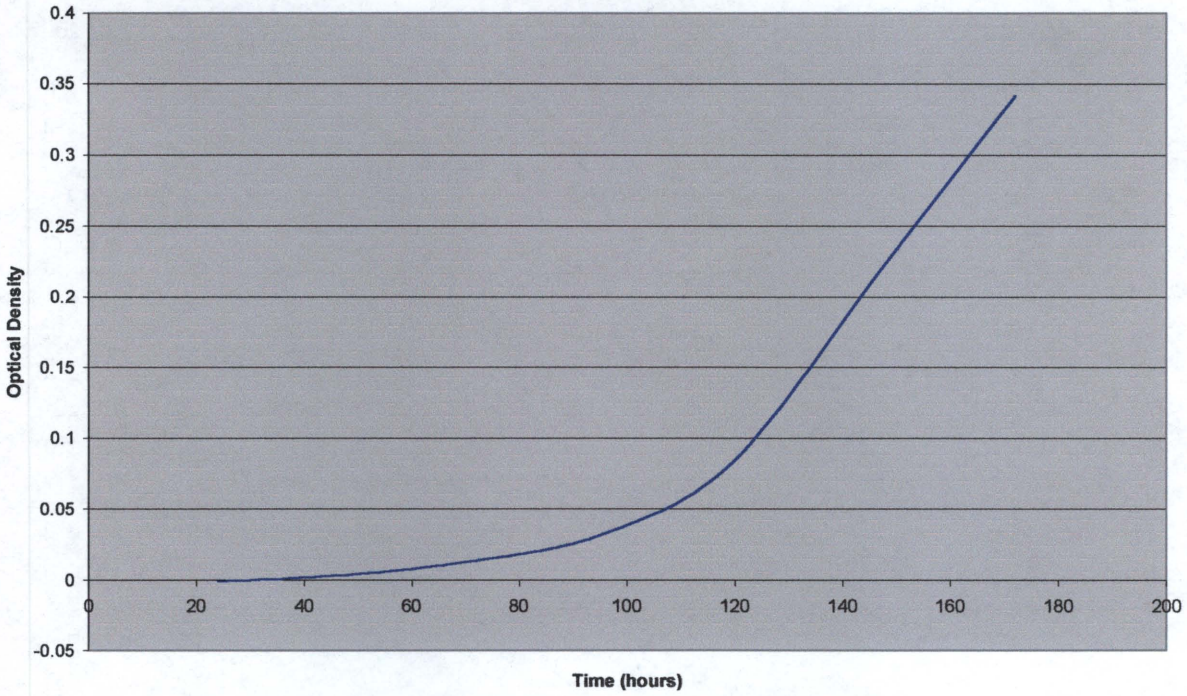
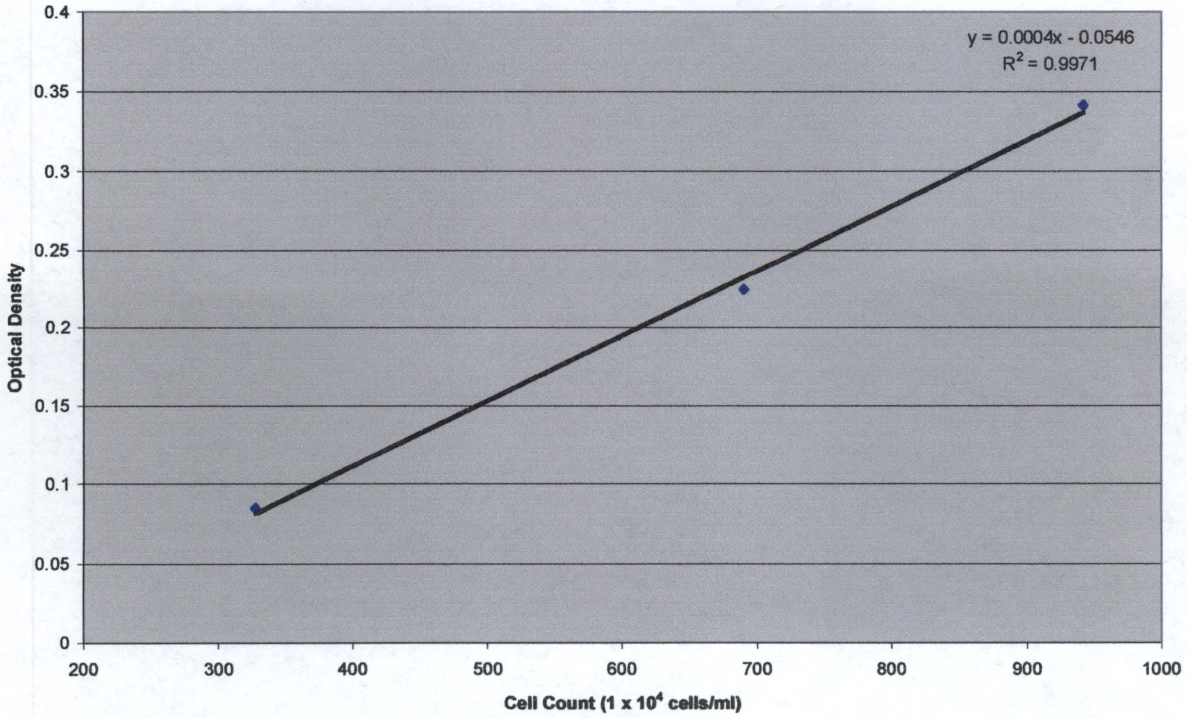


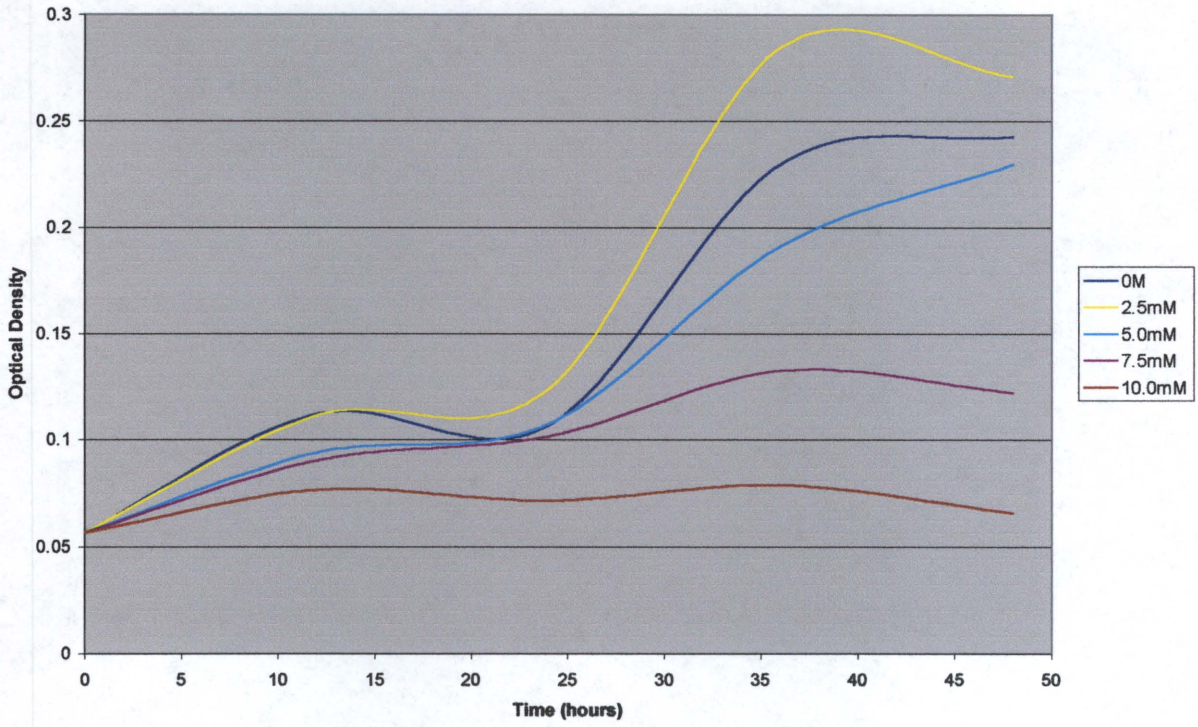
Figure 3



3.2 *Chlamydomonas* phenol growth analysis conditions

Phenol growth analysis studies were conducted in order to determine the phenol concentration to be used in the treatment condition of microarray analysis (Figure 4). The kill curve trials were conducted by growing *C. reinhardtii* on a range of phenol concentrations. There was significant difference between the control (O mM phenol) and *C. reinhardtii* grown in 7.5 mM phenol ($P < 0.05$) on the final day of measurements ($t = 48$ hrs). It is necessary to select a phenol concentration for microarray analysis that does not result in the typical growth pattern of *C. reinhardtii*. The algae must show some growth response to phenol exposure. However, at very high concentrations of phenol, it is likely that the algae are utilizing numerous and complex pathways in response to the environmental stress just to stay alive. Consequently, 7.5 mM was chosen as the phenol concentration for microarray experiments. *C. reinhardtii* grown at this concentration exhibited a growth pattern different from the control group grown at 0 mM phenol, but without the drastic, limiting effects on growth seen at 10 mM phenol.

Figure 4. C. reinhardtii growth when exposed to phenol



4. Discussion

A common method of monitoring *C. reinhardtii*'s growth rate over time is through the use of a hemacytometer and microscope [4]. Recent research has regularly utilized a hemacytometer to take cell density measurements of *C. reinhardti* [5,6,7]. However, this process is frequently time-consuming and laborious when many cultures are grown at once. The growth curve trials were conducted in order to determine the reliability of O.D. as a measure of *C. reinhardtii* growth over time. It was found that between 0.05 and 0.5 absorbance, O.D. measurements of *C. reinhardtii* cultures correlated linearly with standard cell density measurements using a hemacytometer ($r^2 = 0.9971$). The O.D. of up to 96 culture samples can be taken simultaneously using a microplate reader and a 96-well plate. Thus, this finding

provided a more rapid and efficient method of determining algae growth rate during the phenol growth analysis experiments.

The *C. reinhardtii* growth analysis trials revealed that 7.5 mM phenol exposure was the appropriate concentration to be used in microarray analysis. At this concentration, *C. reinhardtii* growth was significantly different from growth at 0 mM phenol. At 7.5 mM phenol, it is likely that the promoters of many genes have been induced by exposure to the environmental stress. Microarray analysis is a technique used to simultaneously observe changes in the transcription of thousands of genes and identify these inducible promoters [8]. First, RNA must be isolated from control and treatment groups that are to be compared, such as a sample grown normally versus one that has been exposed to phenol. The RNA extraction will be done with TRI Reagent, which allows for total RNA isolation and separation of undegraded mRNA [9]. mRNA is the product of transcription. Comparison of mRNA production between control and treatment genes allows identification of genes that are more or less transcriptionally active in the treatment as compared to the control. The mRNA is converted into the more stable complementary DNA (cDNA) with the reverse transcriptase polymerase chain reaction (RT-PCR) technique. Different color fluorescent dyes are used to label the cDNA from each group. For example, the control may be labeled with green dye and the treatment labeled with red dye.

Microarray probes consist of different cDNA or oligonucleotide (small sequence of nucleotides) sequences attached to a glass microscope slide that can hybridize with cDNA. The microscope slides and probes are commercially prepared and contain nucleotide sequences from the genome of the organism of interest. The cDNA targets are mixed and placed onto the slide. During hybridization, the complementary bases in the cDNA targets and probes pair together. The slide is washed to remove unbound cDNA and subsequently scanned with a laser to detect

the bound cDNA. The resulting fluorescent intensities provide information on the relative quantities of cDNA, and consequently of the mRNA of interest. Different quantities of hybridized cDNA in the control and treatment groups would indicate a transcriptional response.

Microarray analysis will be used to observe the effects of phenol on *C. reinhardtii* at the genomic level. Different environmental factors, such as phenol exposure, can affect the rate at which transcription proceeds. For example, if a chemical up regulates transcription of a gene, an increased amount of protein may be produced. In contrast, if gene transcription is down regulated, the corresponding protein may be produced in decreased quantities.

Promoters that are induced as a result of phenol exposure may be revealed through microarray analysis. *C. reinhardtii* grown on liquid TAP medium only will serve as the control. It will be compared to *C. reinhardtii* grown on 7.5 mM phenol/TAP. If *C. reinhardtii* is demonstrated to respond to phenol at the genetic level, future genetic engineering could produce an algae species biosensor for this chemical. A biosensor must be able to indicate in some way that it has detected phenol. A visual response through the use of a reporter gene would be useful. A good candidate would be the green fluorescent protein (GFP), a protein found in jellyfish. GFP fluoresces green (508nm) when exposed to ultraviolet light (395 nm) or blue light (465 nm) [10]. If this gene was fused to a phenol inducible promoter from *C. reinhardtii*, the fluorescence of GFP would predict the presence of phenol in the environment. This technology could be utilized for early detection of water contamination problems and prevention of human health hazards.

Acknowledgements

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