ENHANCEMENT OF AZO DYE DECOLORIZATION BY *Dunaliella tertiolecta* WITH CONVENTIONAL CARBON SOURCES

by

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ENHANCEMENT OF AZO DYE DECOLORIZATION BY *Dunaliella tertiolecta* WITH CONVENTIONAL CARBON SOURCES

In this study, the decolorization of Dye Bath (DB), and the three hydrolyzed dyes, namely Reactive Black 5 (RB-5), Reactive Orange 69 (RO-69) and Reactive Yellow 37 (RY-37) by marine microalgae *Dunaliella tertiolecta*, in a batch culture was investigated and the ability to utilize the azo dyes in the presence of co-substrates namely glucose and glycerol as an additional carbon and energy sources was discussed. Decolorization of dyes was determined by monitoring the decrease in the intensity of absorption at the maximum wavelength (λ_{max}) of each of the dye solution. The color change and pH values of the culture media were monitored during the growth period of algae exposed to dyes in the presence and absence of glucose and glycerol.

The individual and combined effects of DB, RB-5, RO-69 and RY-37 in the presence and absence of glucose and glycerol on the growth of *Dunaliella tertiolecta* were also examined. Algal growth was followed through optical density (OD_{750}) measurements of the culture medium. Furthermore, algal growth was examined with different concentrations of glucose and glycerol.

D. tertiolecta was found to be quite effective in the decolorization of all dyes. At low dilutions (30% and 60% (v/v)) of all DB, RB-5, RO-69 and all dilutions of RY-37 can be removed without co-substrate by the algae. However, addition of both concentrations of glucose and glycerol has some beneficial effects such as increasing the decolorization efficiency or decreasing decolorization period of all dyestuffs except RY-37. At concentrations beyond the IC_{25} values of dyes, decolorization achievement is decreasing due to the different structure and complexity of dyes. Considering the average 8-d IC_{25} and SC_{20} values expressed as % dilution (v/v), the order of toxicity was found as DB>RB-5>RO-69>RY-37. Glucose and glycerol increased the stimulatory effect of all the tested chemicals on the growth of algae at a low concentration range, whereas the inhibitory effect at a high concentration range decreased. Glucose and glycerol did not affect the initial pH values and pH-changing trend of the medium containing dyestuffs, significantly.

EK KARBON KAYNAKLARI İLE *Dunaliella tertiolecta* TARAFINDAN AZO BOYALARININ RENK GİDERİMİNİN SAĞLANMASI

Bu çalışmada, boya banyosu ve üç azo boya çözeltisinin, Reaktif Siyah 5, Reaktif Sarı 37 ve Reaktif Turuncu 69, tuzlu su algi *D. tertiolecta* ile renk giderimi araştırıldı ve algin ek enerji ve karbon kaynağı olarak glikoz ve gliserol varlığında boyayı kullanma kapasitesi tartışıldı. Boyaların renginin giderilmesi, her boya çözeltisinin maximum dalga boyundaki absorbanslarının azalması takip edilerek belirlendi. Ayrıca glikoz ve gliserol varlığında ve sadece boyaya maruz kalan algin büyüme periyodu süresince, renk değişimi ve kültür ortamının pH değerleri de takip edildi.

Boya Banyosu (DB), Reactive Black 5 (RB-5), Reactive Orange 69 (RO-69) and Reactive Yellow 37 (RY-37)un glikoz ve gliserol varlığında ve yokluğunda *Dunaliella tertiolecta*' nın büyümesi üzerindeki etkileri araştırıldı. Algin büyümesi, kültür ortamının 750 nm' deki optik yoğunluğunun ölçülmesi ile takip edildi. Ek olarak, algin büyümesi farklı glikoz ve gliserol konsantrasyonları ile denendi.

Bütün boyaların renk gideriminde *D. tertiolecta* etkili bulundu. Alg düşük seyreltme oranlarındaki boya çözeltisi, RB-5 ve RO-69 ve bütün seyreltme oranlarındaki RY-37'nin rengini ilave substrat olmadan giderilebildi. Diğer taraftan, her iki konsantrasyondaki glikoz ve gliserolün eklenmesinin, RY-37 hariç tüm boyaların renk giderim sürelerinin azalması veya daha fazla renk giderim verimi göstermesi gibi bazı yararları oldu. Sekiz günlük IC_{25} ve SC_{20} değerlerinin seyreltme oranı baz alınarak hesaplanan ortalama değerleri göz önüne alındığında, toksisite sıralaması DB>RB-5>RO-69>RY-37 şeklinde bulundu. Alg için glikoz ve gliserol test edilen tüm kimyasalların düşük konsantrasyon aralığında stimulan etkisini artırdığı ve yüksek konsantrasyonlarda ise inhibisyon etkisini azalttığı görüldü. Glikoz ve gliserol boya içeren kültür ortamının başlangıç pH değerlerini ve değişimini etkilemedi.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation
AUC	Area Under the Curve
CV	Coefficient of Variation
DB	Dye Bath
IC_{25}	The Concentration of the Tested Substance That Decrease the Growth by 25 %
Ig (%)	Per cent Growth Inhibition/Stimulation
OD_{750}	Optical Density at 750 nm
RB-5	Reactive Black 5
RO-69	Reactive Orange 69
RY-37	Reactive Yellow 37
SC_{20}	The Concentration of the Tested Substance That Increase the Growth by 20%
TU	Toxic Unit
Y	Mean Value of Area Under the Growth
Y’	The Lowest Observed Effective Concentration

1. INTRODUCTION

Wastewater from textile, paper and printing industries and dye houses is characterized by a high chemical and biological oxygen demand (COD and BOD), due to the extensive use of synthetic dyes, suspended solids and intense color (Correia et al., 1994). Considering both volume and effluent composition, the wastewater generated by the textile industry is rated as the most polluting among all industrial sectors (Mohan et al., 2002). This is due to the presence of several stages of textile manufacturing where impurities are removed and the required qualities are imparted (Nemerrow, 1987). Dyes used in the textile industry usually have a synthetic origin and complex aromatic molecular structures, which make them more stable and more difficult for biodegradability (An et al., 2002). Dyes are classified into different chemical and application classes according to their variation in application class and chemical nature (Mohan et al., 2002). The structural diversity of dyes is attributed to the presence of different chromophoric groups like azo, triphenylmethane and phthalocyanine (Gill et al., 2002). Azo dyes, the most widely used chemical class in the textile industry, are xenobiotic compounds characterized by the presence of one or more azo linkages (- N = N -) and aromatic rings (Abadulla et al., 2000; Stolz, 2001).

The textile sector alone consumes about 60% of the dyes used for coloration of various fabrics and out of it; around 10-15% of the dyes used for coloration come out through effluents (Abadulla et al., 2000). Release of azo dyes into the environment from the effluents of the industries, has become a major concern in wastewater treatment because some azo dyes are toxic for the aquatic biota as they may be mutagens or carcinogens (Chen, 2002; Chen et al., 2003).

Color is the first contaminant to be recognized in the textile wastewater and has to be removed before discharging to the receiving environment. The presence of very small amounts of dyes in water is highly visible and affects the aesthetic merit, water transparency and the gas solubility in bodies of water (Banat et al., 1997). Colored water is not aesthetically acceptable by the public, and furthermore, color prevents penetration of sunlight into the water and reduces light transmission that could affect primary

productivity. Meaning that, color negatively affects photosynthetic activity and which is required for aquatic life. Color may also diminish visibility necessary for organisms to feed or reproduce (Springer, 1985).

Various treatment methods physical, physico-chemical, biological and chemical processes have been investigated for treating dye-bearing effluents. Physical and chemical methods such as chemical coagulation and ozonation have been used to achieve decolorization of wastewater (Mohan et al., 2002). However, implementation of these methods may generate a significant amount of sludge or may easily cause secondary pollution due to excessive chemical usage or economic unfeasibility (Mohan et al., 2002). On the other hand, biological decolorization and degradation of dyes are relatively cost effective as well as eco-friendly. However, conventional biological treatment systems such as activated sludge and lagoons fail to remove color from the textile industry wastewater due to the complex aromatic structures of the dye (Banat et al., 1997). Anaerobic reduction and decolorization most often generate aryl amines, which are generally more toxic than their parent compounds (Dubrow et al., 1996). No specific process seems to be able to handle decolorization of all textile wastewaters and generally, a customized process; probably involving a combination of methods could be more applicable (Banat et al., 1997).

Currently the focus is on technologies of the treatment of polluted environments that are both cost effective and eco-friendly. Physico-chemical approaches are often cost prohibitive while biological methods are relatively cost effective as well as eco-friendly (Mohan et al., 2002). Although decolorization is a challenging process for the textile industry, microbial decolorizing systems show great potential for achieving total color removal with only hours of exposure (Vijaya and Sandhya, 2003).

Most azo-dye degrading microorganisms such as white-rot fungi, bacteria and algae, cleave the azo bonds of the respective azo dye and produce decolorized products (Wong et al., 2000). The ability of wood-rotting fungi to degrade a wide range of synthetic chemicals, including dyes, has been reported in several studies (Bhatt et al., 2002; Cing and Yeşilada, 2004; Gallagher et al., 1997). They can mineralize xenobiotic materials to CO_2 and H_2O through their highly oxidative and non-specific ligninolytic system

(Chivukula and Renganathan, 1995; Paszcynski et al., 1992). Bacterial degradation of azo dyes is carried out most anaerobically with only a few strains being capable of degradation under aerobic conditions. In both cases colorless and possible toxic aromatic amines are formed. The white-rot fungi have been reported to efficiently degrade azo dyes without the formation of aromatic amines (Schliephake et al., 1993).

On the other hand, there are few studies on decolorization of textile dyes with algae species (Acuner and Dilek, 2004; Aziz and Ng, 1994; Jingi and Houtian, 1992; Guolan et al., 2000; Saçan and Balcıoğlu, 2000). The dye reduction is related to the molecular structure of the dyes and the species of algae used (Mohan et al., 2002). Salt content of textile effluents are high. In general, the quantity varies from 5 to 60 g L^{-1} (Muthukumar and Selvakumar, 2004). Of the recent studies, the marine alga *D. tertiolecta* has been reported to degrade a synthetic dye bath to some extent (Saçan and Balcıoğlu, 2000*)*. The alga has a high salt tolerance (Bakalova and Panova, 2003) ranging from 0.05 to 3.0 mol L^{-1} NaCl (Jahnke and White, 2003). Therefore, it is logical to use this organism for the color removal of synthetic dye bath, which contains high salt concentration in countries like Turkey, surrounded by seas from three sides.

Photoautotrophic microorganisms like algae can grow using CO_2 as carbon and light as an energy source. It is not necessary to supply any organic carbon sources for removal of low concentrations of dyes (Hirooka et al., 2006). On the other hand, co-substrates like glucose, starch and sucrose as an external organic carbon source have been added to enhance the decolorization performance of biological systems in several studies (Haug et al., 1991; Charliell et al., 1995; Kapdan et al., 2000; Sumathi and Manju, 2000).

In this study, our purpose is to investigate the decolorization of a synthetic dye bath including six reactive dye stuffs (Reactive Black 5, Reactive Red 23, Reactive Yellow 37, Reactive Orange 69, Reactive Red 171 and Reactive Blue 209) and additives (urea, NaCl, Na_2CO_3 and NaOH), and the three hydrolyzed dyes having the highest concentration in the dye bath recipe by marine microalgae, *Dunaliella tertiolecta*, in a batch culture and to discuss their ability to utilize the azo dyes in the presence of different concentrations of glucose and glycerol as carbon and energy sources. Growth conditions represent an important factor influencing the efficiency of algae to degrade synthetic dyes. The toxicity

of dyes on the growth of algae and their intermediary products plays a crucial role in dye decolorization and degradation due to the fact that decolorization is growth associated. Furthermore, the individual and combined effects of dyes and co-substrates (glucose and glycerol) on the growth of *Dunaliella tertiolecta* were also examined.

2. THEORETICAL BACKROUND

2.1. Textile Industry

The textile industry is one of the most water demanding sectors. Its consumption can reach from 25 to 250 m^3 of water per ton of product, depending on the processes, the final quality of the product, the type and form of the fibers and the machinery and the equipment employed (Chacon et al., 2005). Water is used extensively throughout textile processing operations, and water consumption varies widely among unit operations (EPA, 1996). Textile finishings, wastewaters, especially dye house effluents contain different classes of organic dyes, chemicals and auxiliaries, thus they are colored and have extreme pH, COD, BOD and AOX values, and they contain different salts, surfactants, heavy metals, mineral oils, and others. Therefore, dye-bath effluents have to be treated before being discharged into the environment or municipal treatment plant (OGRS, 1996).

2.1.1. Process Applied in Textile Industry

The textile industry is comprised of a diverse, fragmented group of establishments that produce and process textile related products for further processing into apparel, home furnishings, and industrial goods. In its broadest sense, the textile industry includes the production of yarn, fabric, and finishing goods. The U.S. Environmental Protection Agency (EPA) has grouped the industry into various categories representing the different industrial activities. The EPA characterization and typical characteristics of wastewater by each of the activities are given in Table 2.1 (Venceslau and Tom., 1994).

In terms of waste generation and environmental impacts, wet processing is the most significant textile operation. Figure 2.1 represents typical wet processing steps for fabrics. Methods used vary greatly depending on end-products and applications, site-specific manufacturing practices, and fiber type. Natural fibers typically require more processing steps than manmade fibers. For most wool products and some manmade and cotton products, the yarn is dyed before weaving; thus, the pattern is woven into the fabric.

Processing methods may also differ based on the final properties desired, such as tensile strength, flexibility, uniformity, and luster (Snowden- Swan, 1995).

Table 2.1. Textile processing categories (Venceslau and Tom., 1994).

Parameter	**Categories**						
	1	2	3	4	5	6	7
BOD_5 ($mg\ L^{-1}$)	6000	300	350	650	350	300	250
TSS ($mg\ L^{-1}$)	8000	130	200	300	300	120	75
COD ($mg\ L^{-1}$)	30000	1040	1000	1200	1000	1000	800
Phenol ($mg\ L^{-1}$)	1.5	0.5	-	0.04	0.24	0.13	0.12
Sulphide ($mg\ L^{-1}$)	0.2	0.1	8	3	0.2	0.14	0.09
Color (ADMI)	2000	1000	-	325	400	600	600
pH	8	7	10	10	8	8	11
Water usage ($1kg^{-1}$)	36	33	13	113	150	0.9	150

1- Raw wool scouring

2- Yarn and fabric manufacturing

3- Wool finishing

4- Woven fabric finishing

5- Knitted fabric finishing

6- Carpet manufacturing

7- Stock and yarn dyeing and finishing

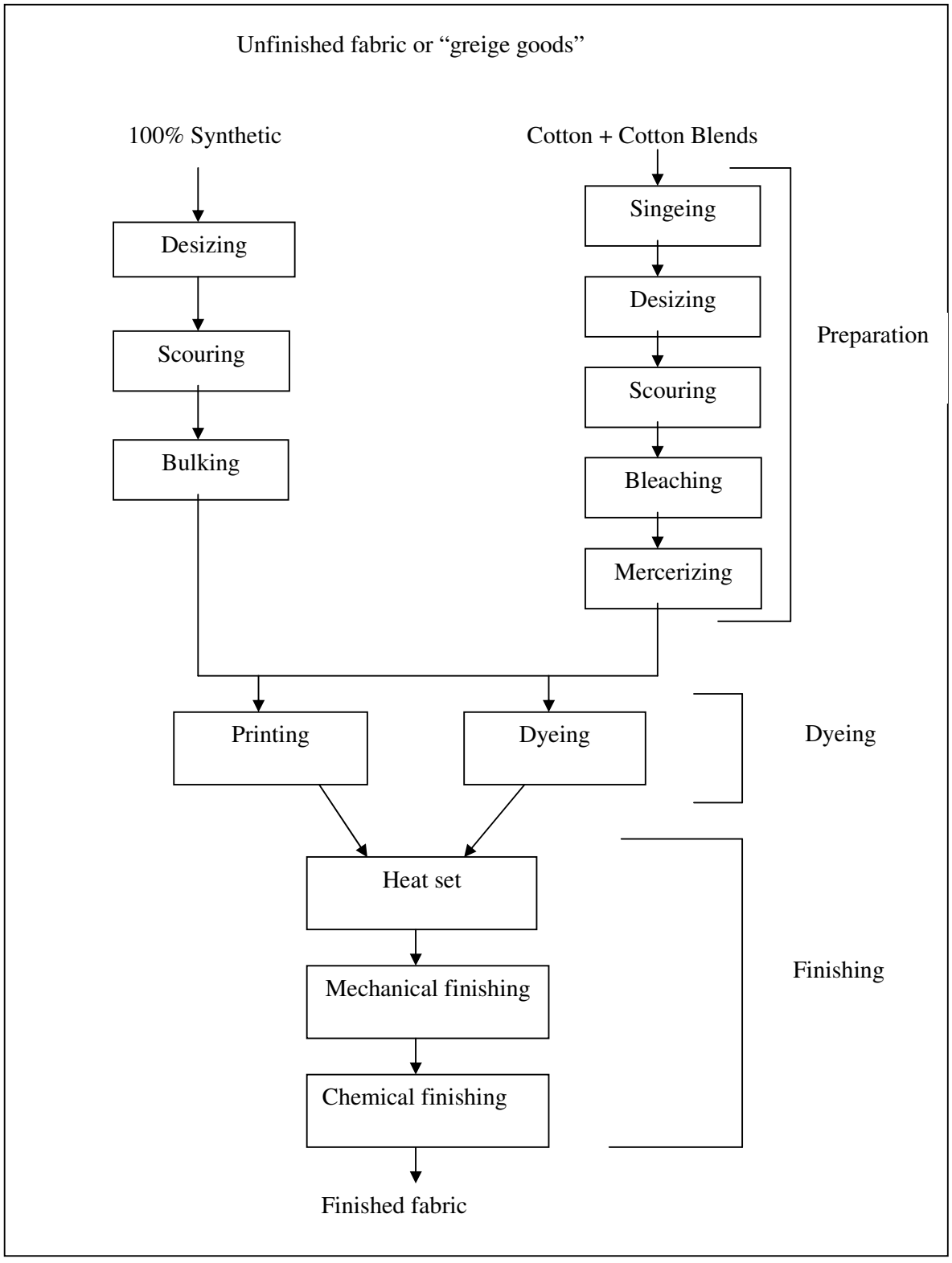


Figure 2.1. Typical wet processing steps for fabrics (Venceslau and Tom., 1994).

The main processes applied in manufacturing can be explained as follows;

Singeing: If a fabric is to have a smooth finish, singeing is essential. Singeing is a dry process used on woven goods that removes fibers protruding from yarns or fabrics. Passing the fibers over a flame or heated copper plates burns these off. Singeing proves the surface appearance of woven goods and reduces pilling. It is especially useful for bricks that are to be printed or where a smooth finish is desired. Pollutant outputs associated with singeing include relatively small amounts of exhaust gases from the burners.

Desizing: Desizing is an important preparation step used to remove sizematerials applied prior to weaving. Manmade fibers are generally sized with water-soluble sizes that are easily removed by a hot-water wash or in the scouring process. Natural fibers such as cotton are most often sized with water-insoluble starches or mixtures of starch and other materials. Enzymes are used to break these starches into water-soluble sugars, which are then removed by washing before the cloth is scoured. Removing starches before scouring is necessary because they can react and cause color changes when exposed to sodium hydroxide in scouring.

Scouring: Scouring is a cleaning process that removes impurities from fibers, yarns, or cloth through washing. Alkaline solutions are typically used for scouring; however, in some cases solvent solutions may also be used. Scouring uses alkali, typically sodium hydroxide, to break down natural oils and surfactants and to emulsify and suspend remaining impurities in the scouring bath. The specific scouring procedures, chemicals, temperature, and time vary with the type of fiber, yarn, and cloth construction. Impurities may include lubricants, dirt and other natural materials, water-soluble sizes, antistatic agents, and residual tints used for yarn identification. Typically, scouring wastes contribute a large portion of biological oxygen demand (BOD) loads from preparation processes (NC DEHNR, 1986). Desizing and scouring operations are often combined (ATMI, 1997).

Bleaching: Bleaching is a chemical process that eliminates unwanted colored matter from fibers, yarns, or cloth. Bleaching decolorizes colored impurities that are not removed by scouring and prepares the cloth for further finishing processes such as dyeing or printing. Several different types of chemicals are used as bleaching agents, and selection depends on

the type of fiber present in the yarn, cloth, or finished product and the subsequent finishing that the product will receive. The most common bleaching agents include hydrogen peroxide, sodium hypochlorite, sodium chlorite, and sulfur dioxide gas. Bleaching contributes less than 5 percent of the total textile mill BOD load (NC DEHNR, 1986). Peroxide bleaching can be responsible for wastewater with high pH levels. Because peroxide bleaching typically produces wastewater with few contaminants, water conservation and chemical handling issues are the primary pollution concerns.

Mercerizing: Mercerization is a continuous chemical process used for cotton and cotton/polyester goods to increase dye ability, luster, and appearance. This process, which is carried out at room temperature, causes the flat, twisted ribbon-like cotton fiber to swell into a round shape and to contract in length. This causes the fiber to become more lustrous than the original fiber, increase in strength by as much as 20 percent, and increase its affinity for dyes.

Dyeing: Dyeing operations are used at various stages of production to add color and intricacy to textiles and increase product value. Textiles are dyed using a wide range of dyestuffs, techniques, and equipment. Dyes used by the textile industry are largely synthetic, typically derived from coal tar and petroleum-based intermediates.

Printing: Fabrics are often printed with color and patterns using a variety of techniques and machine types. Of the numerous printing techniques, the most common is rotary screen. However, other methods, such as direct, discharge, resist, flat screen semicontinuous), and roller printing are often used commercially. Compared to dyes, pigments are typically insoluble and have no affinity for the fibers. Resin binders are typically used to attach pigments to substrates. Solvents are used as vehicles for transporting the pigment and resin mixture to the substrate.

*Finishing***:** Finishing encompasses chemical or mechanical treatments performed on fiber, yarn, or fabric to improve appearance, texture, or performance. Mechanical finishes can involve brushing, ironing or other physical treatments used to increase the luster and feel of textiles. Application of chemical finishes to textiles can impart a variety of properties ranging from decreasing static cling to increasing flame resistance. The most common

chemical finishes are those that ease fabric care, such as the permanent-press, soil-release, and stain-resistant finishes.

2.1.2. Main Wastewater Sources and Effluent Characteristics

Wastewater is, by far, the largest wastestream for the textile industry. Large volume wastes include washwater from preparation and continuous dyeing, alkaline waste from preparation, and batch dye waste containing large amounts of salt, acid, or alkali. Primary sources of biological oxygen demand (BOD) include waste chemicals or batch dumps, starch sizing agents, knitting oils, and degradable surfactants. Wet processing operations, including preparation, dyeing, and finishing, generate the majority of textile wastewater.

Types of wastewater include cleaning water, process water, noncontact cooling water, and stormwater. The amount of water used varies widely in the industry, depending on the specific processes operated at the mill, the equipment used, and the prevailing management philosophy regarding water use. Because of the wide variety of process steps, textile wastewater typically contains a complex mixture of chemicals and the list of chemicals is listed in Table 2.2.

Desizing: The process of removing size chemicals from textiles is one of the industry's largest sources of wastewater pollutants. In this process, large quantities of size used in weaving processes are typically discarded. Desizing processes often contribute to up 50 percent of the BOD load in wastewater from wet processing (Snowden-Swan, 1995).

Bleaching: Bleaching wastewater usually has a high solid content with low to moderate BOD levels (Table 2.3). The dissolved oxygen content of this effluent can be usually high due to the decomposition of hydrogen peroxide. On the other hand, chloride and hydrogen peroxide can cause toxicity problems in biological treatment processes (Venceslau, 1994).

Mercerizing: Mercerization wastewater has low BOD and TS but it is highly alkaline prior to neutralization. The low BOD content comes from surfactants and penetrating agents used as auxiliary chemicals.

Table 2.2. Most frequent auxiliary chemicals in dyeing (Buckley 1992).

Description	Composition	Function
Salts	Sodium chloride	Neutralize the zeta potential of the fiber; Retarder-
	Sodium sulfide	
Acids	Acetic and sulphuric acids	pH control
Bases	Sodium hydroxide	pH control
	Sodium carbonate	
Buffers	Phosphate	pH control
Sequetering agent	EDTA	Complex hardness; Retarder
Surface active agents	Anionic, cationic and nonionic	Disperse dyes; Regulate dye application; Softener
Oxidizing agents	Hydrogen peroxide	Insoluble dyes
Reducing agents	Sodium hydrosulphite	Solubilize dye; Remove unreacted dye

Dyeing: Dyeing operations generate a large portion of the industry's total wastewater. The primary source of wastewater in dyeing operations is spent dyebath and washwater. Such wastewater typically contains by-products, residual dye, and auxiliary chemicals. Additional pollutants include cleaning solvents, such as oxalic acid. Of the 700,000 tons of dyes produced annually worldwide, about 10 to 15 percent of the dye is disposed of in effluent from dyeing operations (Snowden-Swan, 1995). However, dyes in wastewater may be chemically bound to fabric fibers (ATMI, 1997). The average wastewater generation from a dyeing facility is estimated at between one and two million gallons per day. Dyeing and rinsing processes for disperse dyeing generate about 12 to 17 gallons of wastewater per pound of product. Similar processes for reactive and direct dyeing generate even more wastewater, about 15 to 20 gallons per pound of product (Snowden-Swan, 1995).

Finishing: Finishing processes typically generate wastewater containing natural and synthetic polymers and a range of other potentially toxic substances (Snowden-Swan, 1995). Pollution from peroxide bleaching normally is not a major concern. In most cases,

scouring has removed impurities in the goods, so the only by-product of the peroxide reaction is water. The major pollution issues in the bleaching process are chemical handling, water conservation, and high pH. Hazardous waste generated by textile manufacturers results primarily from the use of solvents in cleaning knit goods (ATMI, 1996). Solvents may be used in some scouring or equipment cleaning operations, however, more often scouring processes are aqueous-based and cleaning materials involve mineral spirits or other chemicals (ATMI, 1997). Spent solvents may include etrachloroethylene and trichloroethylene (NCDEHNR, 1986).

Table 2.3. Pollution loads of textile wet operations (Venceslau and Tom., 1994).

Fiber	Process	pH	BOD	TS	Water usage
Cotton	Desizing	-	1700-5200	16000-32000	3-9
	Scouring	10-13	50-2900	7600-17400	26-43
	Bleaching	8.5-96	90-1700	2300-14400	3-124
	Mercerizing	5.5-9.5	45-65	600-1900	232-308
	Dyeing	5-10	11-180	500-14100	8-300
Wool	Scouring	9-14	3000-4000	1129-64448	46-100
	Dyeing	4.8-8	380-2200	3855-8315	16-22
	Washing	7.3-10.3	4000-11455	4830-19267	334-835
Nylon	Scouring	10.4	1360	1882	50-67
	Dyeing	8.4	368	641	17-33
Acrylic	Scouring	9.7	2190	1874	50-67
	Dying	1.5-3.7	175-2000	833-1968	17-33
	Final scour	7.1	668	1191	67-83
Polyster	Scouring	-	500-800	-	25-42
	Dyeing	-	480-27000	-	17-33
	Final scour	-	650	-	17-33

2.1.3. Wastewater Characteristics

Provost (1992) agreed with Smith (1989) to divide waste in four different types: hard-to-treat, highly dispersible, hazardous and toxic, and large volume wastes. Each of these four waste types can be found in the textile industry, and they all have their specific characteristics. Treatment is easiest if each waste is considered separately before being combined.

Difficult-to-treat wastes may include dyes, metals, phenols, toxic compounds and/or phosphates. This type of waste is resistant to conventional biological treatment, can pass through the treatment system ending up in the receiving stream where it sometimes causes toxic effects. It is therefore important to minimize through chemical substitution the use of chemicals which result in difficult-to-treat wastes. If no useful substitute can be found for the problem chemical(s), the chemical(s) should be reused, recycled or segregated from the main waste stream and treated separately.

2.1.4. Types of Dyes Used in Textile Industry

Dyes are small molecules consisting of two key components: the chromophore, responsible for the color, and the functional group, which bonds the dye to fiber. Dyes may be classified according to chemical structure or by their usage or application method (Kirk-Othmer, 1979). Table 2.4 lists the major dye classes and the type of dyes, and of fibers for which they have an affinity (EPA, 1995; Swan, 1995).

Acid dyes are water-soluble unionic compounds applied to the nylon, wool, silk, and some modified acyrlic textiles in an acidic medium. They have one or more sulfonic or carboxylic acid groups in their molecular structure (Yang and McGarrahan, 2005).

Reactive dyes are water soluble, anionic dyes that require relatively simple dying methods. They are mainly used for dying cellulosic fiber such as cotton and rayon, but are also used for wool, ilk, nylon and leather. Reactive dyes contain chromophoric groups such as azo, anthraquinone, triarylmethane etc. and reactive groups e.g. viniyl sulphone, and chlorotriazine etc. that form covalent bonds with the fiber.

Table 2.4. Typical Characteristics of Dyes Used in Textile Dyeing Operations (EPA, 1995; Swan, 1995).

Dye Class	Characteristics	Associated Fibers	Typical Fixation (%)	Typical Pollutants Associated with Various Dyes
Acid	Water-soluble anionic	Wool, nylon	80-93	Color; organic acids; unfixed dyes
Basic	Water-soluble cationic, very bright	Acrylic some polyesters	97-98	N/A Neyin kısaltılmışı aşağıya yazalım
Direct	Water-soluble, anionic, poor wet fastness	Cotton, rayon, other cellulosics	70-95	Color; salt; unfixed dye; cationic fixing agents; surfactant; defoamer; leveling and redarding agents; finish; diluents
Disperse	Colloidal dispersion, low water solubility, good wet fastness	Polyester, acetate	80-92	Color; organic acid; carriers; leveling agents; phosohates; defoamers; lubricants; dispersants; delustrants; diluents
Reactive	Water-soluble, anionic, good wet fastness	Other synthetics	60-90	Color; salt; alkali; unfixed dye; surfactants; defoamers; diluents; finish
Sulfur	Organic compounds containing sulfur or sodium sulfide	Cotton, other cellulosics	60-70	Color; alkali; oxidizing agent; reducing agent; unfixed dye
Vat	Water-soluble, anionic, good wet fastness	Cotton, other cellulosics	80-95	Color; alkali; oxidizing agents; reducing agents

Azo dyes contain at least one nitrogen-nitrogen (N=N) double bond, however many different structures are possible. (Zollinger, 1991). Monoazo dyes have only one N=N double bond, while diazo and triazo dyes contain two and three N=N double bonds, respectively. The azo groups are generally connected to benzene and naphthalene rings, but can also be attached to aromatic heterocycles or enolizable aliphatic groups (Zollinger, 1991). These side groups are necessary for imparting the color of the dye, with many different shades and intensities being possible (McCurdy, 1991). A common example of an azo dye can be seen in Figure 2.2. When describing a dye molecule, nucleophiles are referred to as *auxochromes*, while the aromatic groups are called *chromophores*. Together, the dye molecule is often described as a *chromogen*. The absorption and reflection of visible and UV irradiation is ultimately responsible for the observed color of the dye (Zollinger, 1991).

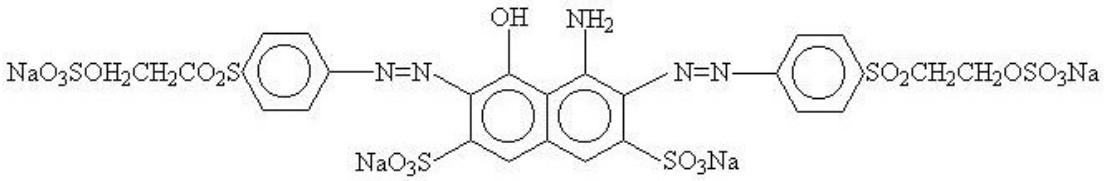


Figure 2.2. Example of an azo dye structure (Reactive Black 5).

Synthesis of most azo dyes involves diazotization of a primary aromatic amine, followed by coupling with one or more nucleophiles. Amino- and hydroxy- groups are commonly used coupling components (Zollinger, 1991). Because of the diversity of dye components available for synthesis, a large number of structurally different azo dyes exist and are used in industry (McCurdy, 1991). World wide production of organic dyes is currently estimated at nearly 450,000 tons, with 50,000 tons being lost in effluents during application and manufacture (Lewis, 1999).

There are a number of different classes of azo dyes, but this study will focus only on fiber-reactive azo dyes. 80 to 95% of all reactive dyes are based on the azo chromogen (Zollinger, 1991; Edwards, 2000). Reactive dyes are colored compounds that contain one or two functional groups capable of forming covalent bonds with the active sites in fibers. A carbon or phosphorous atom of the dye molecule will bond to hydroxyl groups in

cellulose, amino, thiol, and hydroxyl groups in wool, or amino groups in polyamides (Zollinger, 1991; Kirk-Othmer, 1979).

2.1.5. Effects of Textile Wastewater

Enormous volumes of effluent are generated at different stages of textile manufacturing, as a result of the use of copious amounts of chemicals and dyes. Effluent derived from the textile and dye stuff activities can provoke serious environmental impact in the neighboring receptor water bodies because of the presence of toxic reactive dyes, chlorolignin residues and dark coloration. Nature has demonstrated its capacity to disperse, degrade, absorb or otherwise dispose of unwanted residues in the natural sinks of the atmosphere, waterways, ocean and soil. It is realized however that this ability is not finite. The discharge of these waste residues into the environment eventually poison, damage or affect one or more species in the environment, with resultant changes in the ecological balance (Asamudo et. al., 2005).

The dyes present in textile effluent impart persistent color to the receiving streams and interfere with photosynthesis of the phytoplankton (Cunningham and Saigo, 2001). Other physical characteristics of the wastewater include odor, change in dissolved oxygen, presence of insoluble substances and corrosive properties. The colloidal and suspended purities cause turbidity in the receiving streams. The dissolved minerals may increase salinity of the water and thus may render it unfit for irrigation or consumption. Toxic chemicals such as chromium and sulphites may destroy fishes and microorganisms responsible for self-purification of water in streams. Immediate oxygen demand due to the impurities such as starch, sulphites, nitrites, deplete the dissolve oxygen content of water. Starch cotton debris constitute organic wastes which are oxygen demanding. They can undergo decomposition/degradation by bacterial activity. The chemicals use in the processes may change pH of the effluent and once disposed into the water body affects aquatic lives. Dissolved solids can also form incrustations on the surfaces of sewers and chemicals may cause corrosion of the metallic parts of the sewage treatment plants.

Textile effluents can seep into the aquifer and pollute the underground water, or where it is discharged without proper treatment into water bodies, the pollutants cannot be

confined within specific boundaries. They can therefore affect aquatic life in enormous ways. Metallic effluents can have ecological impacts on water bodies leading to increased nutrient load especially if they are essential metals. These metals in effluent may increase fertility of the sediment and water column and consequently leading to euthrophication, which in open waters can progressively lead to oxygen deficiency, algal bloom and death of aquatic life. Water contaminated with metallic effluent can cause several health problems (Asamudo et al., 2005).

2.2. Treatment Methods Applied to Textile Dye-house Wastewater

In the textile industry, production is carried on different processes by using natural and synthetic raw materials such as wool and cotton. The variety of raw materials, chemicals, processes and also technological variations applied to the processes cause a complex and dynamic structure of environmental impact of the textile industry. These dynamic structures not only affect the characterization and quantity of wastewater and the applied purification technologies but also make it impossible to focus on a typical kind of wastewater and a standard purification technology. The basic goal of these purification technologies is for the dispersion of the impurities out of the wastewater by one or a combination of physical, chemical or biological methods.

2.2.1. Physical and Chemical Methods

The physical and chemical techniques were numerous and included physicochemical flocculation combined with floatation, electro-flotation, flocculation with Fe (II)/Ca (OH_2) membrane-filtration, electro kinetics coagulation, electrochemical destruction, ion-exchange, irradiation, precipitation, ozonation, adsorption, and the katox treatment method involving the use of activated carbon and air mixtures (Lin and Liu, 1994; Hosono et al., 1993; Aplin and Waite, 2000).

Surfactants and dyes with high molecular weights are successfully removed by the coagulation/flocculation processes followed by sedimentation, flotation and filtration, respectively. The main advantage of the conventional processes like coagulation and flocculation is decolorization of the waste stream due to the removal of dye molecules

from the dyebath effluents, and not due to a partial decomposition of dyes, which can lead to an even more potentially harmful and toxic aromatic compound. The major disadvantage of coagulation/flocculation processes is the production of sludge. Advantages and disadvantages of the current methods of dye removal from industrial effluents are indicated in Table 2.5.

No one specific process seems to be able to handle decolorization of all textile wastewaters and generally, a customized process, probably involving a combination of method could be more applicable (Banat et al., 1997).

2.2.2. Biological Methods

Several biological treatment systems such as, aerobic activated sludge (Jing and Bishop, 1994), aerobic-anaerobic packed-bed reactors (Schliephake et al., 1993), aerobic-anaerobic fluidized-bed reactors (Seshadri et al., 1994), aerobic-anaerobic sequential batch or continuous flow reactor (Loyd et al., 1992), anaerobic batch reactors (Charliell et al., 1995), have all been used toward the treatment of textile industry wastewaters.

2.2.3. Adsorption by Living/Dead Microbial Biomass

The uptake or accumulation of chemicals by microbial mass has been termed biosorption (Hu, 1992, 1996; Tsezos and Bell, 1989; Kumar et al., 1998). Dead bacteria, yeast and fungi have all been used for the purpose of decolorizing dye-containing effluents.

Textile dyes vary greatly in their chemistries, and therefore their interactions with micro-organisms depend on the chemistry of the microbial biomass (Polman and Brekenridge, 1996). Depending on the dye and the species of micro-organism used different binding rates and capacities can be observed. It can be said that certain dyes have a particular affinity for binding with microbial species.

The use of biomass has its advantages, especially if the dye containing effluent is very toxic. Biomass adsorption is effective when conditions are not always favorable for the growth and maintenance of the microbial population (Modak and Natarajan, 1995).

Table 2.5. Advantages and disadvantages of the current methods of dye removal from industrial effluents (Robinson et al., 2001).

Physical/ chemical methods	Advantages	Disadvantages
Fenton's reagent	Effective decolorization of both soluble and insoluble dyes	Sludge generation
Ozonation	Applied in gaseous state: no alteration of volume	Short half-life (20 min)
Photochemical	No sludge production	Formation of by-products
NaOCl	Initiates and accelerates azo-bond cleavage	Release of aromatic amines
Cucurbituril	Good sorption capacity for acid dyes	High cost
Electrochemical destruction	Breakdown compounds are non-hazardous	High cost of electricity
Activated carbon	Good removal of wide variety of dyes	Very expensive
Peat	Good adsorbent due to cellular structure	Specific surface areas for adsorption are lower then activated carbon
Wood chips	Good sorption capacity for acid dyes	Requires long retention times
Silica gel	Effective for basic dye removal	Side reactions prevent commercial application
Membrane filtration	Removes all dye types	Concentrated sludge production
Ion exchange	Regeneration: no adsorbent loss	Not effective for all dyes
Irradiation	Effective oxidation at lab scale	Requires a lot of dissolved O_2
Electro kinetic coagulation	Economically feasible	High sludge production

Adsorption by biomass occurs by ion exchange. Biosorption tends to occur reasonably quickly; a few minutes in algae to a few hours in bacteria (Hu, 1996).

2.3. Recent Studies on the Decolorization of Dyes

There are several studies on decolorization of dyes by various microorganisms. Some of these studies can be summarized as follow;

Numerous bacteria capable of dye decolorization have been reported in literature. Effects to isolate bacterial cultures capable of degrading azo dyes started in the 1970's with reports of a *Bacillus subtilis* (Horitsu et al., 1977), then *Aeromonas hydrophila* (Idaka and Ogawa, 1978). They were followed by a *Bacillus cercus* (Wuhrmann et al., 1980). An *azo reductase* enzyme was responsible for the degradation of the Orange II dye by *Pseudomonas* strains and substituting any of the groups near the azo groups chemical structure inhibiting the degradation (Zimmermann et al., 1982).

In the study by An et al., (2002), *A. citrobacter sp.,* isolated from soil at an effluent treatment of a textile and dying industry, decolorized several recalcitrant dyes except Bromophenol Blue. More than 90% of Crystal Violet and Methyl Red were reduced within 1 h. Gentilen Violet, Malachite Green and Brillant Green lost over 80% of their colors in the same conditions. Decolorization of Congo Red was mainly due to adsorption to the cells. Color removal was optimal at pH 7-9 and 35-40°C. Decolorization of dyes was also observed with an extracellular culture filtrate, indicating the color removal by enzymatic biodegradation.

Chen et al., (2003) isolated six bacterial strains with the capability of degrading textile dyes from sludge samples and mud lakes to observe decolorization of the textile dyes. *Aeromonas hydrophila* was selected and identified because it exhibited the greatest color removal from various dyes. Although *A. hydrophila* displayed good growth in an aerobic and agitation culture, color removal was the best in anoxic and anaerobic culture. Nitrogen sources such as yeast extract or peptone could enhance strongly the decolorization efficiency. In contrast to a nitrogen source, glucose inhibited decolorization activity

because the consumed glucose was converted to organic acids that might decrease the pH of the culture medium, thus inhibiting the cell growth and decolorization activity.

The degradation of two azo dyes; Congo Red and Direct Black 38, in use in the textile industries of Turkey, were investigated using two facultative microorganisms (*Escherichia coli and Pseudomonas sp.*) under aerobic, anaerobic and microaerophilic conditions by Işık and Sponza (2003). Simultaneous biomass activity and color removal performance were monitored during batch assays. The effects of two different microorganisms and aerobic/anaerobic conditions on decolorization were recorded with monitoring of color, pH, COD, dissolved oxygen, alkalinity and volatile fatty acids concentrations. The color of the CR and DB 38 dyes were removed up to 98 and 72% by *E. coli* at the end of anaerobic incubation, while no decolorization occurred throughout the aerobic incubation. Under microaerophilic conditions, the azo dyes CR and DB 38 were removed up to 39 and 75% by *E coli*. In studies with *Pseudomonas sp* the color of the CR and DB 38 dyes were removed up to 100 and 83% after five days of anaerobic incubation, while 76% and 74% color removal efficiencies were observed under microaerophilic conditions.

According to the study by Song et al., (2003), *Rhodobacter sphaeroides* decolorized more than 90% of several azo dyes in 24 hours. The optimal culture condition was anaerobic illumination, peptone as a carbon source, temperature 35-40°C and pH 7-8. Addition of a carbon source accelerated decolorization of Red Dye II. The decolorization rate with peptone reached 95% in 24 hours which was much faster than the others. Methyl Orange was used as the experimental dye in enzyme assays.

The work by Dias et al., (2003) examined the extracellular enzymatic system of strain Euc-1. Its ability to decolorize 14 xenobiotic azo dyes was evaluated and compared with the well–known species *Phanerochaete chrysosporium.* It was found that laccase activity increased with the temperature rise of up to 50-60°C. Solid plate decolorization studies showed that *Basidiomycete* Euc-1 decolorized 11 azo dyes, whereas *Phanerochaete chrysosporium* decolorized only two of them.

The study, by Chang et al., (2004), provides an attempt to use the extracellular metabolites of a dye-decolorizing strain, *Escherichia coli* strain NO_3, as a biostimulater to entice *E. coli* strain NO_3 into a beneficial mode of metabolism for an economically feasible decolorization. Substantial decreases in oxidation-reduction potential to negative levels were observed during anaerobic decolorization in the presence of the metabolite. Results of repeated batch cultures also showed that serial acclimation of *E. coli* strain NO_3 significantly increased decolorization ability. The metabolite produced from higher dye-concentration decolorization led to more enhancement of the bacterial decolorization.

In addition several fungi have been shown to be capable of decolorization. Chang et al., (2001) designed and tested three different reactor configurations for decolorization of the azo dye, Orange II, with white rot fungus. All the bioreactors showed high and stable decolorization activity in long-term operation.

Decolorization of four synthetic azo dyes was examined in two white rot fungal cultures by Chagas and Durrant, (2001). In a solidified culture medium, *P. chrysosporium* partially decolorized all the tested dyes, while *P. sajorcaju* totally decolorized amaranth, new coccine, and orange G, but not tatrazine. In liquid culture medium, *P. chrysosporium* totally decolorize amaranth, new coccine, and Orange G, and 60% tatrazine, *P. sajorcaju* totally decolorize amaranth, new coccine, 50% orange G, and a maximum of 20% tatrazine.

Nyanhonga et al., (2002) screened four ligninolytic fungi, *Trametes modesta, Trametes hirsuta, Trametes versicolor and Sclerotium rolfsii* for their ability to decolorize eight synthetic dyes (anthraquinone, azo, indigo and triarylmethane) by producing laccase. The decolorization rate depended on the both source of the enzyme preparation and on the structure of the dye. Based on laccase production and dye decolorization ability, *T. modesta* was selected for further studies. All the tested dyes were decolorized by the *T. modesta* under acidic conditions (pH 3-6) and at 50-60°C.

Irpex lacteus, a cosmopolitan white fungus, has been studied in connection with proteinase and cellulase production, lignin degradation and degradation PAH in shallow stationary cultures by Kasinath et al., (2003). Good capacity for decolorization of azo,

anthraquinone, phthalocyanine and triphenylmethane dyes by *I. lacteus* in stationary liquid cultures has been demonstrated. The fungus was also able to degrade an anthraquinone dye in contaminated soil.

The white fungus, *Fomes lividus*, was tested for decolorization of azo dyes such as Orange G, Congo Red, Amido Black and also for color removal from dye industry effluents by Selvam et al., (2003). The result showed that the fungus could remove only 31% of Orange G in the synthetic solution, whereas Congo Red and Amido Black were removed by 74 to 99%, respectively.

In addition several yeasts have been shown to be capable of decolorization. A number of simple azo dyes were degraded in liquid aerated batch cultures by a strain of the yeast *Candida zeylanoides* by Martins et al., (1999). The standard decolorization medium contained glucose as a carbon and energy source. The extent of color removal in the culture medium was assessed through the decrease in dye absorbance of the supernatants. The extent of color removal ranged from 44 to 90%, after seven days, for five out of six dyes studied in shake cultures.

According to the study by Yang et al., (2003), two yeasts, *Debaryomces polymorphus, Candida tropicalis,* and two filamentous fungi, *Umbelopsis isabellina, Penicillium geastrivorus*, could completely decolorize 100 mg Reactive Black 5 within 16-48 h. Manganese-dependent peroxidase activities between 60 and 424 U I^{-1} were detected in culture supernatants of three of these organisms indicating the color removal by enzymatic biodegradation but with *Penicillium geastrivorus* there was no ligninolytic enzyme activity in its culture and the decolorization was mainly dye biosorption to mycelium. Extensive decolorization by *D. polymorphous* and *C. topicalis* was obtained with five other azo dyes and one anthraquinone dye.

2.4. Algal Treatment of Textile Dyehouse Wastewater

2.4.1. Algal Cultures as Treatment Technology

The algae are a diverse grouping of plants that crop up in a wide range of environmental habitats. Most crop up in the fresh and salt waters that cover over 70% of the earth's surface, but they also crop up in the soil, on land plants, in permanent ice sheets, and snowfields. They are photosynthetic plants that contain chlorophyll, have simple reproductive structures, and their tissues are not differentiated into true roots, stems, or leaves.

Taxonomists have placed the algae into a number of major groups, or divisions, depending on certain characteristics. The commonly occurring algae are members of the following divisions: Bacillariophyta (diatoms), Charophyta (charophytes), Chlorophyta (green algae), Chrysophyta (yellow-green, or yellow-brown algae), Cryptophyta (cryptomonads), Cyanophyta (blue-green algae), Euglenophyta (euglenoids), Phaeophyta (brown algae) Pyrrhophyta (dinoflagellates), and Rhodophyta (red algae). At times references are made to the algae by these group names, so it is helpful to be familiar with them.

The algae display a great variety of growth forms. They range from unicellular or single cells, to fairly complex multicellular organisms. Some unicellular species are motile and can move around. Multicellular species display a variety of life forms; colonial and filamentous organization are two common examples. Colonies are aggregations of a few to many individual algal cells growing in association with one another. Filaments are strands of cells that can be either branched or unbranched. Some algae have such a complex growth form that they are mistaken for vascular plants - members of the charophyte group are one example.

The size of individual algal plants range from microscopic, unicellular species which are approximately 0.000039 in. (0.0010 mm) in diameter to large filamentous marine algae that obtain lengths of over 100 ft (30 m).

It has been estimated that the marine algae account for more than 90% of the world's photosynthetic activity, making them the most important source of oxygen (Bold and Wynne, 1985).

Like all organisms, the algae require certain conditions for growth; light, temperature, and the availability of inorganic nutrients are three major conditions. All algae require the radiant energy of sunlight to carry on the processes of photosynthesis. For different species there is a broad range of optimal light conditions. Some have high light requirements, while others only need minimal amounts of radiant energy for growth. Temperature is another important growth requirement. In fresh water the optimum temperature for growth of most algae lies between 59° F (15° C) and 77° F (25° C). However, some algae can actively grow at 32° F (0° C) while others grow in thermal springs or geysers where water temperatures are up to 185° F (85° C). In addition, the different requirements of various species for certain nutrients are an important factor for determining which species will be found in abundance in a given body of water.

The algae are an essential component of all aquatic systems since they serve as the base of the food chain for all other organisms. In terrestrial settings they are important members of the soil flora. In purification plants, water supply reservoirs, and sewage treatment plants they play a primary role in oxygenation and filtration

Microalgal cultures offer interesting alternatives for wastewater as secondary or tertiary treatment to remove inorganic nutrients, while producing potentially valuable biomass for the growth of microalgae, effluents from secondary domestic wastewater treatment plants and from anaerobically-digested animal wastes can be well suited, since they contain high amounts of inorganic nutrients and relatively low amounts of organic compounds (Travieso et al., 1996).

Gonzales et al., (1997) studied the removal of ammonia and phosphorus from Colombian agro-industrial wastewaters by the microalgae *C. vulgaris* and *Scenedesmus dimorphus*. In this study, the main aim is to find microalgal species with a high efficiency of ammonia and phosphorus removal, and to compare the efficiency of the removal in two bioreactor types as cylindrical and triangular. For this purpose, *C. vulgaris* and

Scenedesmus dimorphus were selected. As a result, it was found that usage of C. *vulgaris* in the triangular reactor was superior for removing ammonia and the cylindrical bioreactor for removing phosphorus was superior.

Furthermore, Wong et al., (2000) studied nickel biosorption by two chlorella species, *C. vulgaris* and *C. miniota*. Nickel-zinc, and copper-nickel-chromium removals by *C. vulgaris* is examined by Chang et al., (1999), respectively.

As a conclusion, the wastewater treatment by microalgae cultures has a major advantage that it does not generate additional pollution when biomass is harvested and it allows efficient recycling of nutrients (Gonzales, 1997).

2.4.2. Algal Treatment of Dyes Used in Textile Industry

Aziz and Ng (1994) studied color removal from synthetic textile wastewater by using an activated algae-reactor. The activated algae-reactor with mixed population of algae and bacteria is intended to treat highly organic and colored, toxic metals and nutrient-loaded wastewaters. A continous flow system containing three rectangular reactors, connected in series, was designed. 22 units of 40 W cool greenhouse fluorescent lamps placed about 40 cm above the tanks. The wastewater contains an azo dye (Brillant Violet 5R), which represents a wide range of dyes used in textile dyeing processes. For both samples, the dye removal efficiency was observed more than 90%. The mechanism for color removal by algae was determined to be adsorption. This was supported by visual observations of the algae-reactors where pink masses of algae were observed.

Jingi and Houtian (1992) examined the degradation of azo dyes by 3 types of algae, namely, *C. vulgaris, Oscillatoria tenuis* and C*hlorella pyrenoidosa*. More than 30 azo compounds with 20-ppm concentration were selected in the decolorization test. This test was continued for 96 h after centrifugation at 5000 rpm, supernatant was evaluated via the light absorption method. As a result, it was found that the azo reductase of *C. vulgaris* is an induced enzyme and the substrate can act as a kind of inducer. The induction causes a marked enhancement of the enzyme activity. In this study, as a last test, algal degradability of aniline formed as aromatic amines after the cleavage of the azo bond was investigated.

As a result of the HPLC analysis, it was found that 100% loss of aniline by means of algal action was reached and no other organic compounds were detected. They proposed a whole degradation pathway of azo compounds by algae.

Guolan et al., (2000) examined the physiology and degradation of dye with immobilized algae. Immobilization of *Chlorella pyrenoidosa* was performed by using calcium alginate. They observed that after immobilization, both algal growth and physiological activity increased. On the cell growth and physiological activity, effects of algal size and initial density were found minor. The resistance of mass transfer of CO_2 was the limiting factor for the distribution of algae inside the support and this distribution was not homogeneous. In addition, algal cell division inside the medium was restricted when bubbling air containing 2% CO_2 was not applied. After bubbling air with 2% CO_2, algal growth and cell division considerably increased and individual cell size restored normally. In the case of the degradation of dye namely Direct Brown NM, immobilized algae reacted with this dye for two days. Decolorization rates for immobilized algae without bubbling of CO_2 were 82% and 65% for dye concentration of 30 mg L^{-1}, respectively. Conversely, free algae reached only 12% decolorization. As a result, it was understood that immobilization increased the decolorization rate for algae especially when there is bubbling air with 2% CO_2. Furthermore, while immobilized algae reacted with azo dye for four days, the efficiency was 81%, coimmobilized with the addition of bacterium efficiency increased to 93% for four days. Hence, it was stated that addition of bacterium increased the anabolic activity of algae.

By Valderrama et al., (2002), laboratory-scale experiment were performed to develop a procedure for biological treatment of recalcitrant anaerobic industrial effluent using first the microalgae *Chlorella vulgaris* followed by the macrophyte *Lemna minuscula.* This recalcitrant dark color wastewater, containing high levels of organic matter and low pH, prevents the growth of microalgae and macrophyte, and therefore, could not be treated by them. Hence the wastewater diluted to 10% of the original concentration with wash water from the production time. *C. vulgaris* population reduced ammonium ion, phosphorus, and COD and dissolved a flowing microbial film after five days of incubation. Consequently, *Lemna minuscula* was able to grow in the treated wastewater and reduced other organic matter and color after an additional six days of incubation. This study demonstrates the

feasibility of combining microalgae and macrophytes for bioremediation of recalcitrant wastewater.

Acuner and Dilek., (2004) aimed to investigate the treatment of a mono-azo dye, namely Tectilon Yellow 2G, with both unacclimated and acclimated cultures of *C. Vulgaris*. COD removal efficiencies were determined as 69, 66 and 63% for the initial TY2G concentrations of 50, 200 and 400 mg/L, whereas acclimation of *C. vulgaris* caused them to increase to 88, 87 and 88% respectively. *C. vulgaris* was also effective in the treatment of TY2G, especially when acclimatized. The mechanism of TY2G removal was not adsorption onto the algal biomass, but bioconversion or degradation. TY2G was found to be first converted to aniline, which is further degraded by *C. vulgaris* upon prolonged exposure times in case of unacclimated algae while no aniline was detected in case of acclimated algae.

2.5. Factors Affecting Dye Biodegradation

Due to the highly variable nature of biological treatment systems and especially textile effluents, there are a number of factors that may affect the biodegradation rate of azo dyes. Throughout certain literature, researchers have discussed various problems associated with dye biodegradation that may or may not be anticipated or remedied. Non-dye related parameters such as temperature, pH, dissolved oxygen or nitrate concentrations, bacteria consortium, and cell permeability can all affect the biodegradation of azo dyes and textile effluents. Dye related parameters such as class and type of azo dye (i.e. reactive-monoazo), reduction metabolites, dye concentration, dye side-groups, and organic dye additives could also affect the biodegradability of azo dye wastewaters.

Wuhrmann et al., (1980) investigated the effects of pH, temperature, type and concentration of respiration substrates, and oxygen tension on the rate of biological reduction of a variety of azo dyes by a consortium of microbes, including *Bacillus cereus* and *Sphaerotilus natans.* Temperatures, which are too high or too low, can result in the exclusion of a particular group of microorganisms. Using activated sludge, Wuhrmann *et al*., (1980) determined that temperature has an increasing linear relationship with the reduction rate of Orange II and Lanasyl violet up to 28 ºC. In general however, most

studies have been conducted at set temperatures, offering minimal data on temperature effects.

The wastewater pH can affect the proper functioning of both anaerobic and aerobic organisms (Grady et al., 1999). Wuhrmann et al., (1980) also investigated the effect of pH on dye reduction rates, but he was unable to conclusively establish a relationship. However, they did state that an exponential increase in the decolorization rate was observed by decreasing the pH, but this relationship depended on the dye being tested.

Nitrate and especially oxygen may play an important role in determining the rate of dye reduction. The presence of oxygen generally inhibits the degradation of azo dye chromogens. Interestingly, Wuhrmann et al., (1980) demonstrated that obligate aerobes might actually decolorize azo compounds under temporary anoxic conditions. However, high nitrite or nitrate concentrations in the mixed liquor of activated sludge plants could significantly inhibit dye removal. Zissi and Lyberatos (1996) observed *Bacillus subtilis* to degrade *p*-aminoazobenzene under anoxic conditions.

The type of bacteria or consortium used for dye biodegradation will undoubtedly affect the reduction rate. In general however, aerobic microbes do not have the ability to substantially decolorize azo dyes, but can oxidize the dye metabolites. The converse applies to anaerobic microbes.

A final non-dye related factor is the cell permeability and the cell wall adsorption of azo dyes. Wuhrmann et al., (1980) investigated the effects of dye absorption by the cell wall and concluded the following: (1) dye adsorption follows Freundlich adsorption isotherms at low dye loads per weight of biomass, but exhibits a high variability; (2) depending on the dye, subsequent reduction may take place or the dye may remain in the cell wall; (3) adsorption does not inhibit the reduction rate of microbes that exhibit the ability to reduce azo dyes.

Cell permeability might play an important role in dye biodegradation. In the study conducted by Wuhrmann et al., (1980), all dyes that were not reduced by whole cells were effectively degraded by cell extracts from both facultative anaerobes and obligate aerobes.

This suggests that many cells might be capable of dye biodegradation, but are limited by the permeability of their cell walls.

The effectiveness of microorganisms in decolorizing azo-dyes depend on the structure and complexicity of the dyes, particularly on the nature and position of substituent in the aromatic rings and the resulting interactions with the azo bond. In general, the more azo linkages that must be broken will cause the reduction rate to be slower. Brown and Laboureur (1983) observed that two poly-azo dyes showed only moderate to variable biodegradation as compared to four mono azo and six diazo dyes. The authors indicate that poly-azo dyes are less likely to be degraded than mono- or diazo dye types.

The production of toxic by-products or the presence of toxic dye additives may also inhibit biodegradation. High salt concentrations are not uncommon in textile effluents and may result in adverse conditions for biodegradation. Dispersing and solubilizing agents may also create inhibitory conditions for dye reduction (Charliell, 1995). O'Neill et al., (1999) concluded from respiration- inhibition testing that anaerobic degradation of simulated textile effluent generated metabolites that were toxic to some aerobic organisms.

A final and important factor to evaluate is the initial dye concentration of the wastewater. Seshadri and Bishop (1994) performed a study investigating the effect of different influent dye concentrations on the color removal efficiency. They concluded that elevated dye concentrations may cause a drop in percent dye removal. Furthermore, the inhibition may be directly related to the effects of increased dye metabolite formation due to higher dye concentrations. Less pronounced reductions were seen at lower concentration levels. It should be noted that tolerable influent concentrations are likely specific to individual or related groups of dyes. Charliell et al., (1995) also states that toxicity assays showed that C.I. Reactive Red 141 was inhibitory to anaerobic organisms at concentrations greater than 100 mg L^{-1}, however prior biomass adaptation increased their resistance to elevated dye concentrations.

2.6. Test Organisms

Biological toxicity testing is applied to organisms at different tropic levels. The test organisms incorporated in these assays include microorganisms, plants, and invertebrates and fish. Most of the aquatic plants including algae are more sensitive than animals to a variety of potential toxicants and also algae are the dominant primary producers in the aquatic food chain. Test assays on various effluents from municipalities and industries have demonstrated greater sensitivity to algae than animal species. Algae and duckweed were more sensitive than invertebrates and fish to several detergent surfactants, textile effluents, acridine, dyes, synfuels, herbicides, and a variety of other phytotoxic compounds (Lewis, 1993; Walsh et al., 1982).

2.6.1. *Dunaliella tertiolecta*

D. tertiolecta is described by Butcher (1959) as an ovoid to ellipsoidal cell 9-11 µm long and 5.5-7 µm wide with an acute apex, tapering anter or, rounded posterior and a thin firm, hyaline periplast. It is bioflagellata unicellular green algae with a cell structure typical of the members of the order volvocales (class *chlorophyceae*) though lacking a cell wall.

Figure 2.3. A cell of *D. Tertiolecta* (http://www. protist.i.hosei.ac.jp/ PDB /Images /Chlorophyta/Dunaliella/).

D. tertiolecta is a naked, unicellular green alga which is well known for its extraordinarily high tolerance to salt stress, high light and relatively high temperatures (Berges and Falkowski, 1998). Although the optimum salinity for *D. tertiolecta* is 0.05 mol L^{-1} which is native to the marine environment it can grow in salinities ranging from 0.05 to

3.0 mol L^{-1}NaCl (Jahnke and White, 2003). Other *Dunaliella* species (*bardawil, parva* and *salina*) grew at higher salinities (5 mol L^{-1}NaCl), but could not acclimate to salinities less than 0.2–0.35 mol L^{-1}. *D. tertiolecta* grow in media with high concentration of $MgSO_4$ where as this species does not grow in media containing $MgCl_2$, $Mg(NO_3)_2$ and LiCl (Fujii, 1991). Members of the genus *Dunaliella* can be found in the Dead Sea, the Great Salt Lake in Utah, some lakes in Wadi Natman in Egypt and many other sites (Oren et al., 1995). The culture of *Dunaliella* used in this study was isolated from the Sea of Marmara by the research group from Scientific and Technical Research Council of Turkey (TÜBİTAK) Marmara Research Center.

2.7. Toxicity Considerations

While this study does not directly address the problem of toxicity created by the release and degradation of azo dyes, consideration of this problem is still warranted. The potential for toxic effects to the environment and humans, resulting form the exposure to dyes and dye metabolites, is not a new concern. As early as 1895 increased rates in bladder cancer were observed in workers involved in dye manufacturing (Rehn, 1895). Since that time, many studies have been conducted showing the toxic potential of azo dyes.

As mentioned previously, azo dyes are primarily composed of aromatic amines. Substituted benzene and naphthalene rings are common constituents of azo dyes, and have been identified as potentially carcinogenic agents. While most azo dyes themselves are non-toxic a significantly larger portion of their metabolites are toxic (Ganesh, 1992). An investigation of several hundred commercial textile samples revealed that nearly 10% were mutagenic in the Ames test (McCarthy, 1997). Another study conducted on 45 combined effluents from textile finishing plants showed that 27% of the wastewater samples were mutagenic in the Ames test (McCarthy, 1997).

Other concerns are for the impurities within commercial dye products and the additives used during the dyeing process. Many textile effluents contain heavy metals that are complexed in the azo dyes. High concentrations of salt are often used to force fiber-reactive dyes out of solution and onto substrates (Zollinger, 1991). These compounds can

cause high electrolyte and conductivity concentrations in the dye wastewater, leading to acute and chronic toxicity problems.

Understanding the dye structures and how they are degraded is crucial to understanding how toxic by-products are created. Brown and De Vito (1993) have compiled a three-part list of the biological mechanisms thought to be responsible for carcinogenic activation of azo dye compounds. This list is based on an extensive review of the literature regarding azo dye toxicity, and places each mechanism in order of their frequency of citation. Brown and De Vito (1993) postulate that:

- Azo dyes may be toxic only after reduction and cleavage of the azo linkage, producing aromatic amines.
- Azo dyes with structures containing free aromatic amine groups that can be metabolically oxidized without azo reduction may cause toxicity.
- Azo dye toxic activation may occur following direct oxidation of the azo linkage producing highly reactive electrophilic diazonium salts.

2.8. Toxicity Tests and Dose-Response Relationship

The USEPA (1995) defines toxicity testing as "the means to determine the toxicity of a chemical or effluent using living organisms. A toxicity test measures the degree of response of an exposed test organism to a specific chemical or effluent". There are many forms and methodologies for a toxicity test; the two most widely used forms of toxicity testing are the acute and chronic toxicity tests performed using both freshwater and marine organisms. **Acute toxicity tests** are short-term tests designed to measure the effects of pollutants on species during a short part of their life span. The tests, which typically run for between 48 and 96 h, usually measure the effects of pollutants on the survival of a species. The results of these tests are often reported as an 'EC_{50}', which is the effective concentration of a test sample that causes a specific effect (here a 50% reduction in survival).

Chronic toxicity tests are used for low-level pollutants and high-risk situations. These are long-term tests relative to the life span of the species, and are designed to measure larval development or growth, and reproductive success or failure (Donald, 1998).

Toxicity assessment is quite complex, many factors can affect the results of toxicity tests. Some of these factors include variables like temperature, food, light, and stressful environmental conditions. Other factors related to the animal itself include age, sex, health, and hormonal status (http://pmeq.cce.edu/profiles/extoxnet/TIB/dose reponse.html.).

The science of toxicology is based on the principle that there is a relationship between a toxic reaction (the response) and the amount of poision received (the dose). An important assumption in this Dose-Response relationship is that there is almost always a dose below which no response occurs or can be measured. As second assumption is that once a maximum response is reached any further increases in the dose will not result in any increased effect. The classical concentration-response relationship is depicted as a sigmoidal shaped (S-shaped) curve; however the particular shape of concentration-response curve may differ for each coupled toxicant and response pair (Calabrese, 2002). Two parameters of this curve are used to describe it:

1- The concentration or dose that results in 50% of the measured effect and
2- The slope of the linear part of the curve that passes through the midpoint. The midpoint is commonly referred to as a LD_{50}, LC_{50}, EC_{50}, and IC_{50}.

The definitions of these parameters can be described as follows (Landis and Yu, 1999):

LD_{50} : The dose that causes mortality in 50% of the organisms tested estimated by graphical or computational means.
LC_{50} : The concentration that causes mortality in 50% of the organisms tested estimated by graphical or computational means.
EC_{50} : The concentration that has an effect on 50% of the organisms tested estimated by graphical or computational means.
IC_{50} : Inhibitory concentration that reduces the normal response of an organism by 50% estimated by graphical or computational means. Growth rates of algae, bacteria, and other organisms are often measured as an IC_{50}.

Generally the concentration-response concept is the basis for the determination of inhibition concentration in toxicity. Inhibition concentration is a point estimate of the

toxicant concentration that would cause a given per cent reduction in a non-lethal, non-quantal biological measurement, such as growth. For example, an IC_{25} is the estimated concentration of a toxicant that would cause a 25% reduction in average growth. Inhibition concentrations may be calculated using a linear interpolation method such as EPA's Bootstrap Procedure.

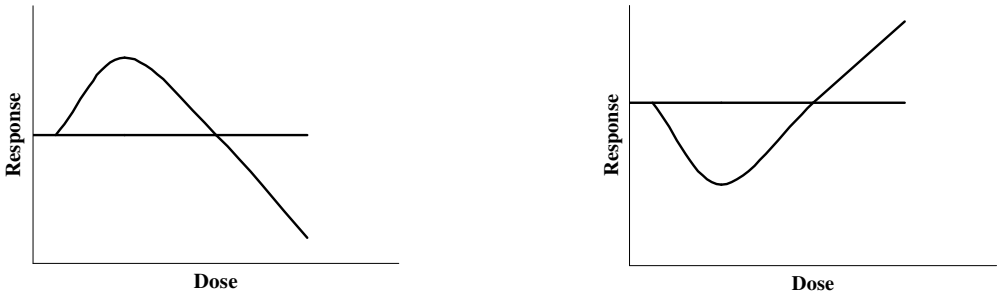
A true assessment of chemical toxicity involves comparisons of numerous dose-response curves covering many different types of toxic effects. Traditionally, regulatory agencies such as the Environmental Protection Agency (EPA), the Food and Drug Administration (FDA), and the Nuclear Regulatory Commission (NRC) use a threshold model for non-carcinogens, and a linear no-threshold model for carcinogens (including radiation)(Figure 2.5). In the threshold model, anything above a certain dose is considered dangerous, and anything below it safe. In the linear model, there is no safe dosage. Changing to a hormesis model would likely change exposure standards for these toxins in air, water, food and soil. As a result, costs of environmental regulations and cleanup/remediation activities could be lowered.

In toxicology, hormesis is a dose response phenomenon characterized by a low dose stimulation, high dose inhibition, resulting in either a J-shaped or an inverted U-shaped dose response. A pollutant or toxin showing hormesis thus has the opposite effect in small doses than in large doses (http/: en.wikipedia.org/wiki/Hormesis.html.).

The quantitative features of the hormetic dose response is a modest stimulation at low doses where the maximum stimulation is typically 30-60% greater than the control, and a range of stimulation that can be variable but is typically less than 10-20 fold, although approximately 5-7% of hormetic dose responses can exceed 100-fold. In fact, so routinely was the hormetic response observed that the investigator proposed the creation of the term SC_{20} (i.e. the stimulation concentration for 20% increase above the controls) to describe the stimulatory response in the low concentration range (Walsh et al., 1982).

(a) (b)

Figure 2.4. Stylized curves illustrating (a) linear, (b) threshold model (Calabrese, 2002).



(a) (b)

Figure 2.5. The most common form of hormetic dose response curves (a) inverted U-shaped, (b) U-shaped (Calabrese, 2002).

3. MATERIALS and METHODS

3.1. The Synthetic Reactive Dye Bath and Single Dye Stock Solutions

In this study synthetically prepared dye-bath and dyes according to procedure obtained from local integrated textile manufacture plant were used. Dyes were supplied from DyStar and Clarient. The synthetic dye-bath contained six reactive dyestuffs widely used in textile industry and proper amounts of assisting chemicals. The concentration of the reactive dyes and auxiliary chemicals in the synthetic stock solutions are summarized in Table 3.1. In order to guarantee that all dyestuff in the prepared mixture were 100 per cent hydrolyzed, they were dissolved in boiling deionized water together with their assisting chemicals and stirred for four h and then the dye-bath and single dye stock solution was kept in a cool and dark conditions for twelve h before use (Arslan, 2000).

3.2. Sea-water

Sea-water was taken in August 2004 and in April 2005 from the coast of Samatya in Istanbul and stored in deep freeze after filtration at –24 ºC in a plastic container. While decolorization tests of Dye Bath, Reactive Black 5, Reactive Orange 69 and Reactive Yellow 37 in the presence and absence of glucose were carried out in sea-water taken in August 2004 (Table 3.3), decolorization tests of dyes with glycerol were carried out in sea-water taken in April 2005 (Table 3.4). Natural sea-water was characterized by measurement of pH, temperature, salinity, conductivity, chloride, alkalinity, nitrate, total kjeldahl nitrogen and phosphate based on standard procedures (APHA, 1998) (Table 3.2-3.3).

3.3. Preparation of Algal Culture

In this study, a unicellular green algae "*D. tertiolecta*" had been used throughout the experiments. Culture of the green algae, *D. tertiolecta*, was supplied by (TÜBİTAK). *D. tertiolecta* was cultured in 500 mL Erlenmeyer flask in a controlled growth chamber (WTCB Binder Model KBF 240) under a continuous light regime, and maintained at a

Table 3.1. The concentration of the chemical components present in the synthetic dye bath and their functions in the dyeing process (Arslan, 2000).

Dyestuff (color index)	**Company**	**Reactive Group**	**Concentration(g L^{-1})**
Remazol Black SB 133% (Reactive Black 5)	Dystar (Hoechst)	Sulphatoethylsulphonate	0.583
Remazol Red 3B (Reactive Red 23)[a]	Dystar (Hoechst)	Sulphatoethylsulphonate	0.084
Remazol Brillant Yellow GL (Reactive Yellow 37)	Dystar (Hoechst)	Sulphatoethylsulphonate	0.126
Drimarene Orange K-GL (Reactive Orange 69)	Clariant (Sandoz)	Monochlorodifluoropyrimidine	0.167
Drimarene Rubinol R/K-5BL (Reactive Red 171)	Clariant (Sandoz)	Monochlorodifluoropyrimidine	0.076
Drimarene Blue K-2RL (Reactive Blue 209)	Clariant (Sandoz)	Monochlorodifluoropyrimidine	0.092
Assisting Chemicals	**Function**		**Concentration(g L^{-1})**
Urea	Increase solubility of the dyestuff		3.0
NaCl	Transfer dyestuff to fabric		70.0
Na_2CO_3 (soda ash)	pH buffer		5.0
NaOH	Produce covalent bonds between dyestuff and fabric		4.0

[a] Contain 1.6% Copper in Complexed Form

Table 3.2. Characterization of sea-water taken from Samatya cost of Istanbul in August 2004.

Parameters	Unit	Value
pH		8.1
Temperature	°C	23.4
Salinity	‰	20.3
Conductivity	mS cm^{-1}	33.9
Cl^-	g L^{-1}	17.4
NO_3^- N	mg L^{-1}	1.5
TKN	mg L^{-1}	10
Alkalinity	mg $L^{-1}CaCO_3$	145
PO_4^{3-}	mg L^{-1}	1.1

Table 3.3. Characterization of sea-water taken from Samatya cost of Istanbul in April 2005.

Parameters	Unit	Value
pH		8.3
Temperature	°C	15.1
Salinity	‰	19.6
Conductivity	mS cm^{-1}	31.8
Cl^-	g L^{-1}	14.7
NO_3^- N	mg L^{-1}	2.8
TKN	mg L^{-1}	12
Alkalinity	mg $L^{-1}CaCO_3$	144
PO_4^{3-}	mg L^{-1}	0.9

Table 3.4. Final concentration of nutrients in culture medium (f/2) and origin of chemical substances used (Okay et al., 1994).

Nutrient	Amount (mg L^{-1})	Concentration (µM)	Origin
$NaNO_3$	75	882.35	Merck
$NaH_2PO_4.2H_2O$	5	23.82	Merck
$Na_2SiO_3.5H_2O$	12.9	60.85	Merck
$CuSO_4.5H_2O$	0.01	0.04	Merck
$ZnSO_4.7H_2O$	0.022	0.09	Merck
$CoCl_2.6\ H_2O$	0.01	0.04	Panreac
$MnCl_2.4\ H_2O$	0.18	1.11	Merck
$FeCl_3.6\ H_2O$	3.15	11.65	Merck
$Na_2Mo_4.2\ H_2O$	0.6	0.13	Merck

temperature of 18±2 °C. Experimental cultures were carried out in natural filtered (0.47 µm GF/C Glass microfiber Whatman filters) sea-water and enriched with f/2 medium (Okay et al., 1994) described in Table 3.4.

Each treatment had a minimum of three replicates plus at least one control. Three types of experiments were done in parallel. In one set, the growth of *D. tertiolecta* was initiated in a sterile medium containing only a chosen dye. In the second and third set of experiments the growth of *D. tertiolecta* was initiated in a medium supplemented with 0.1 g L^{-1} or 0.2 g L^{-1} glucose or glycerol and a chosen dye. Four types of control were disposed: flasks with microalgae but no azo dyes and/or glucose/glycerol (growing controls), flasks with only nutrient solution, flasks with microalga and glucose/glycerol but no azo dyes, flasks with azo dyes and glycerol/glucose but no algae (background controls for glucose/glycerol). Flasks were hand shaken twice daily to prevent settling of the cells. All glassware was washed with nitric acid and rinsed thoroughly with tap water. Deionized water was used for the final rinse. All glassware was sterilized at 180ºC for two hours in a sterilizer. Seawater and nutrient solutions were sterilized by a standard autoclaving procedure.

3.4. Test Medium

Batch experiments were carried out in a 500 mL conical flask and each treatment (including the control) had three replicates. Synthetic dye-bath and single dye stock solution made up 40-fold concentrated in deionized water together with assisting chemicals and were filter sterilized prior to their addition to the sterile culture medium. They were diluted with natural seawater to give a concentration series of 30, 60, and 90% and enriched with a modified f/2 medium (Okay et al., 1994).

3.5. Measurement of Algal Response

Algal response was monitored through optical density measurements of the culture medium using a Hach Spectrophotometer (DR/2010) at 750 nm.

The coefficient of variation (CV) was calculated by dividing the standard deviation by the mean and multiplying by 100 for the purpose of comparison of endpoint variability as follows:

$$\mathrm{CV} = \left(\frac{S.D.}{Y} \right) \times 100 \qquad (3.1)$$

where *S.D.* is the standard deviation and *Y* is the mean value of area under the growth.

3.6. Data Analysis

Growth curves were constructed and the area under the growth curve for individual test vessels was calculated with the following equation (LeBlond and Duffy, 2001):

$$AUC = \frac{(N_1 - N_0)}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \ldots\ldots + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1}) \qquad (3.2)$$

where,

AUC = area under the growth curve,

N_0 = absorbance (750 nm)/fluorescence intensity at the beginning of the test (relative units),

N_1 = absorbance (750 nm)/fluorescence intensity at the selected time interval (relative units),

N_n = absorbance (750 nm)/fluorescence intensity at the end of the selected time interval (relative units),

t_1 = the first selected time, and

t_n = the n times selected times.

% inhibition/stimulation of the growth area of individual test vessels to the mean value for control in each experiment was calculated using the following equation:

$$I_g(\%) = \frac{AUC_c - AUC_t}{AUC_c} \times 100 \qquad (3.3)$$

where,

I_g = per cent inhibition/stimulation of the growth area of an individual test vessel,

AUC_t = area of each test vessel, and

AUC_c = mean area of the controls in each experiment.

(+) Positive percentage indicates inhibition and (-) negative percentage indicates a stimulating effect as the algal density in the waste containing sample is higher than that of the control.

3.7. Statistical Analysis

Dunnett's post-hoc test was employed to determine if treatments were significantly different than the control using the General Linear Model (GLM), univariate procedure in SPSS 11.0 software. The most common parameter used in algal toxicity assays is the IC_{50} or IC_{25}, i.e., the concentration of the tested substance that decreases the growth by 50% or 25%, respectively. IC_{25} values were determined using linear interpolation combined with bootstrapping (known as the IC_p method) as outlined in USEPA (1993) and Noberg-King (1993). The 8-d SC_{20} values were estimated from the curve fitting analysis applied on the data including dye dilution % versus % inhibition/stimulation (Ig). The 8-d IC_{25} values of DB with and without glucose and RB-5 were estimated from the same curves plotted for the estimation of the 8-d SC_{20} values. From the best fitted equation of the developed curves the 8-d SC_{20} values were found using Scientific Workplace-PC+ software. Data were statistically analyzed by the paired *t*-test ($p<0.05$).

The joint toxicity of mixtures is generally evaluated with method based on empirical toxic unit (TU) concepts (Moreau et al., 1999). TU corresponds to the ratio of 100/Y, where Y' corresponds to the lowest observed effective concentration (LOEC). In this study, TU was calculated using both the 8-d IC_{25} and SC_{20}.

3.8. Decolorization Determination

3.8.1. Scanning Absorbance

Decolorization of dyes was followed by monitoring change in its absorption spectrum (200 to 750 nm) using a spectrophotometer (Unicam Helios α-β UV-Vis). For the absorbance measurement of cell free samples, the aliquots collected aseptically from the cultures were centrifuged for 20 min at 10000 rpm (P Selecta-MeditronicBL-5). Decolorization of dyes was determined by monitoring the decrease in the intensity of absorption at the maximum wavelength (λ_{max}) of the each dye solution.

3.8.2. Color

Color of samples was monitored for dilutions ranging from 30% (v/v) to 90% (v/v) to support the changes in the absorption spectra of dyes with *D. tertiolecta* by Hach Spectrophotometer (DR/2010) directly as a platinum cobalt (Pt-Co) unit which is applicable to both domestic and industrial wastewaters (APHA, 1998). Since turbidity must be removed before analysis to determine color by currently accepted methods (APHA, 1998), our samples were centrifuged for 20 min at 10000 rpm (P Selecta-MeditronicBL-5) before the color measurement to remove turbidity interferences. Color removal was calculated from the color values obtained against the control.

3.8.3. pH and Conductivity Measurements: pH (WTW pH 330/SE 1) and conductivity (WTW LF 320/SET) were also measured during eight days.

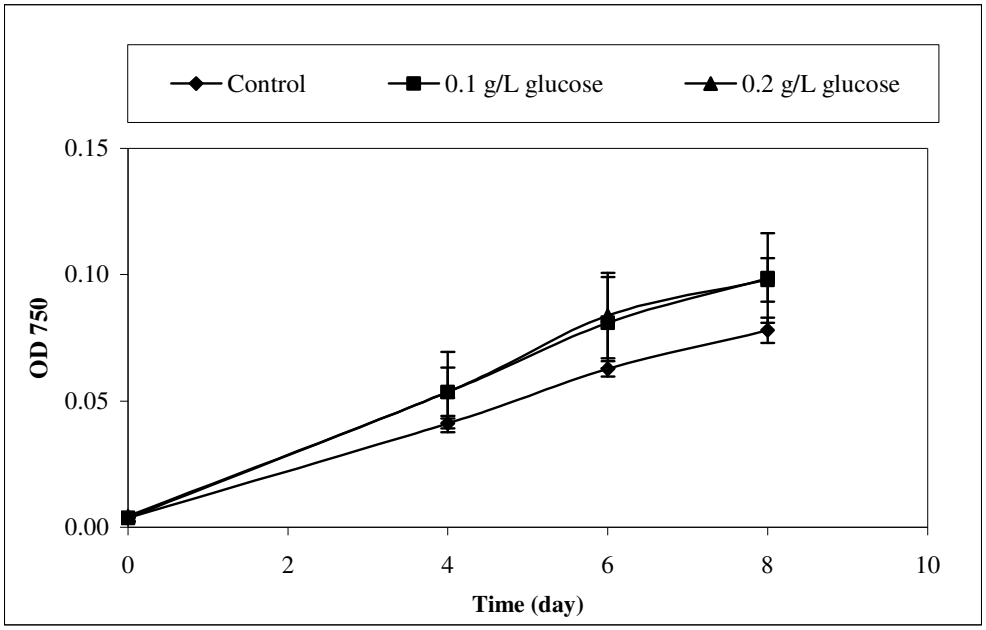
4. RESULTS AND DISCUSSIONS

4.1. Growth Response of *Dunaliella tertiolecta* exposed to Glucose and Glycerol

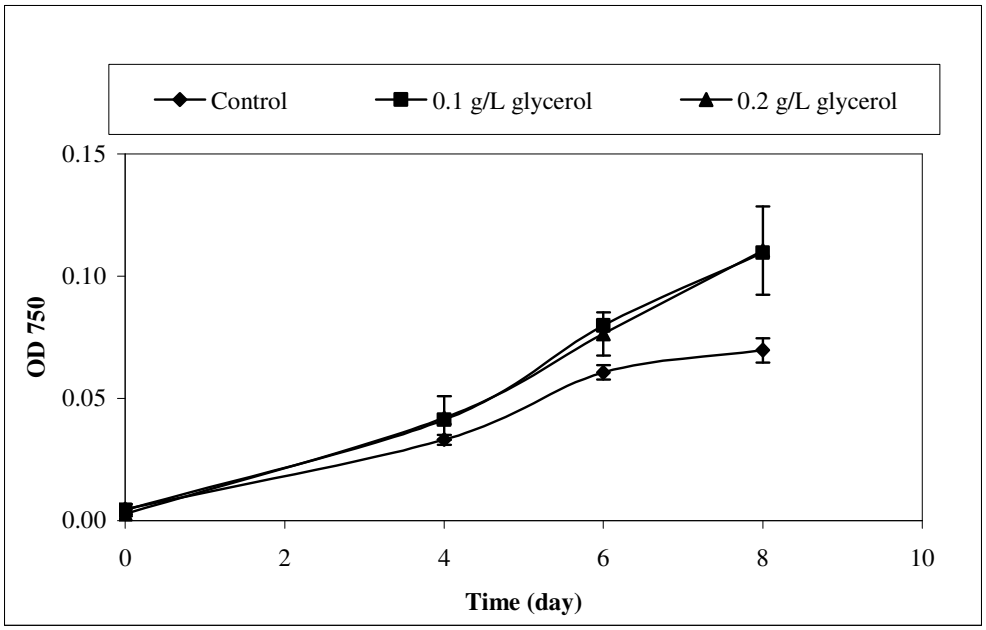
Glycerol and glucose as a carbon and energy source were tested independently for their ability to support the growth of *D. tertiolecta* in test medium. Growth curves of *D. tertiolecta* exposed to different concentrations of glucose and glycerol were given in Figure 4.1. Error bars show the mean standard deviation between triplicates.

Algal growth was observed during the first eight days of incubation. A lag phase duration (four days) was observed to be almost the same at all flasks singly and combined with 0.1 g L^{-1} and 0.2 g L^{-1} glucose and glycerol. It can be said that the lag phase duration is governed by the autotrophic growth process of the algae. It is known that the algal lag phase is highly dependent on the light intensity rather than the organic strength of the medium as long as the nutrients required for the autotrophic growth are available. On the other hand, in the exponential phase, the growth of algae showed an increasing trend with the addition of 0.1 g L^{-1} and 0.2 g L^{-1} glucose and glycerol compared to the control. However, increasing glucose and glycerol concentration from 0.1 g L^{-1} to 0.2 g L^{-1} did not effect the growth of *D. tertiolecta*.

In literature, the effects of glucose and glycerol on the growth of microorganisms are available. Mayo and Noike, (2005) investigated the effect of glucose concentration on the growth of *Chlorella vulgaris* and heterotrophic bacteria in the mixed culture. They found that growth rates of algae and bacteria increased with the glucose-loading rate but excessive loading rates were detrimental to the survival of the algae and bacteria. On the other hand, Massa et al., (2001) reported that as the glucose concentration increases, a decrease of the microbial growth of enterotoxic *E. coli* occurs. In addition, Borowitzka and Brown, (1973) reported that glycerol and glucose enhanced the inhibitory effect of salt on *D. tertiolecta.* Similarly, Mahishi et al., (2002) reported that basal medium supplemented with glycerol as a sole carbon source supported maximum cell growth of *E. coli*. Wood et al., (1998) reported that glycerol seemed to be utilized better than glucose by *Nanochloropsis, Rhodomonas* and *Cyclotella.*



(a)



(b)

Figure 4.1. Growth curves of *D. tertiolecta* exposed to a) glucose and b) glycerol.

al., (1998) reported that glycerol seemed to be utilized better than glucose by *Nanochloropsis, Rhodomonas* and *Cyclotella.*

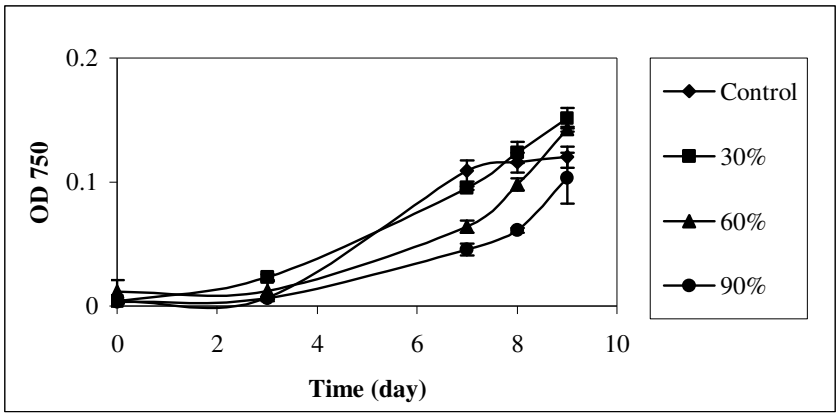
4.2. Dye Bath

4.2.1. Growth of *Dunaliella tertiolecta* Exposed to Dye Bath with and without Glucose

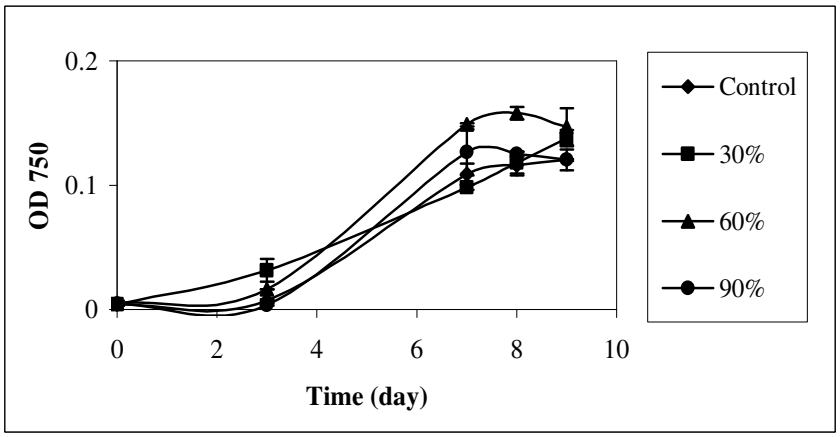
To evaluate the effect of toxicity of the Dye Bath (DB) with and without glucose on the growth of *Dunaliella tertiolecta,* batch studies were conducted with varying dye concentrations (30, 60 and 90% dilutions (v/v)). Growth curves of *D. tertiolecta* exposed to different dilution ratios of DB with 0.1 g L^{-1} and 0.2 g L^{-1} glucose and without glucose were given in Figure 4.2. Error bars show the mean standard deviation between triplicates. All studied dilutions (% (v/v)) revealed a significant difference from the control ($p<0.05$).

Algal growth was observed during the eight days of incubation. Algal growth was promoted at low dilutions of the DB and the biomass increased with the increase in number of days. A lag phase duration (four days) was observed to be almost the same at all dilutions tested (30, 60 and 90% dilutions (v/v)). It can be said that the lag phase duration is governed by the autotrophic growth process of the algae as stated in section 4.1. On the other hand, in the exponential phase, the growth of algae at all dilutions except 30% (v/v) of DB is lower than that of the control. However, addition of both concentrations of glucose resulted in stimulation of the growth of algae for 60% and 90% (v/v) compared to single exposure of DB (Figure 4.2 and Table 4.1). The growth of algae showed a decreasing trend with increasing dye concentration. Reduction in cell growth may result from the toxicity of dyes to algae through the inhibition of metabolic activities. Azo dyes generally contain one or more sulphonic-acid groups on aromatic rings, which might act as detergents to inhibit the growth of microorganisms (Wuhrman et al., 1980). The same authors reported that dyes were the inhibitors for nucleic acid synthesis or cell growth.

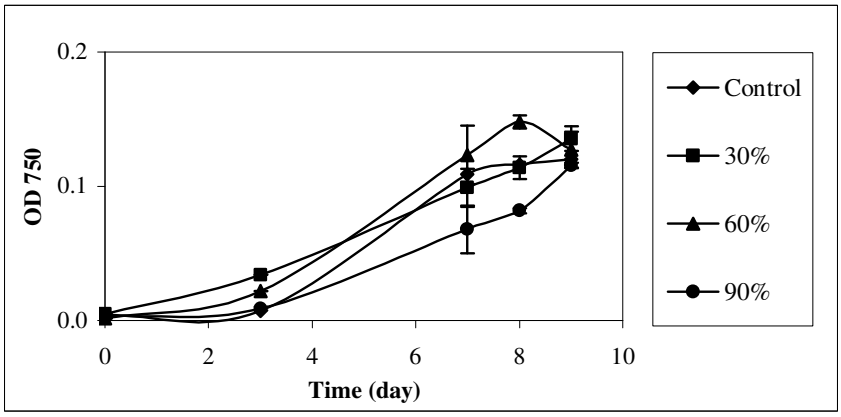
While hormetic response was observed in DB, glucose eliminated the inhibitory effect of DB. Hormesis is a dose-response phenomenon characterized by low-dose stimulation, high-dose inhibition. Hormetic-like biphasic dose-response routinely observed by USEPA scientists**.** Hormetic effects have also been reported in studies dealing with complex mixtures of petroleum (Mommerts 1973, Dunstan 1975, Delistrary 1986,



(a)



(b)



(c)

Figure 4.2. Growth curves of *D. tertiolecta* exposed to a) DB b) DB and 0.1 g L^{-1} glucose and c) DB and 0.2 g L^{-1} glucose.

Nicoletti and Egli, 1998), pharmaceutical effluents (Saçan and Balcıoğlu, 2006) and with a wastewater effluent (Srivastava and Sahai 1987).

Calculated area under the growth curves (AUC) from optical density (OD_{750}) were tabulated in Table 4.1 together with the 8-d IC_{25} and SC_{20} and TU values. The 8-d SC_{20} values of DB, DB with 0.1 g L^{-1} glucose and DB with 0.2 g L^{-1} glucose were in the range of 24.94%, 24.74-78.72% and 12.05-80.31% (v/v), respectively. Inhibitory effect was observed at a higher dilution percentage. DB was toxic to algae with the 8-d IC_{25} values of 54.90±2.22% (v/v). Both concentrations of glucose eliminated the inhibitory effect of DB. In other words, no toxicity of DB with 0.1 g L^{-1} and 0.2 g L^{-1} glucose was detectable with an IC_{25} of >100% (Table 4.1). Therefore, the 8-d IC_{25} values couldn't be calculated for DB in the presence of 0.1 g L^{-1} and 0.2 g L^{-1} glucose. When the 8-d IC_{25} and SC_{20} values of DB were considered, addition of 0.1 g L^{-1} and 0.2 g L^{-1} glucose as a carbon and energy source increased the stimulatory effect of DB significantly ($p<0.05$) compared to single exposure of DB.

Although DB is stimulatory singly at low dilutions and in combination with glucose at high dilutions, it may cause a change in the fine structure of algal cells. Both organic and inorganic toxicants are capable of altering the fine structure of algal cells, with no obvious correlation between this damage by either type of pollutants (Rachlin et al., 1982). Mooney and Patching, (1998) reported that chemostat–grown cells of the chlorophyte *Dunaliella tertiolecta* exposed to triphenyltin underwent structural damage and tyhlakoid membranes of a small proportion of cells spread from the usual compact arrangement. On the other hand, DB at high dilutions is toxic to *D. tertiolecta* when we consider the growth inhibition values (Ig) (Table 4.1). Toxic effects can be seen with a change in the cell permeability. Sikka et al., (1973) demonstrated that the herbicide dichlone alters the cell permeability in *Chlorella* as evidence by a rapid efflux of intracellular material from the cell. Similarly, Parasher et al., (1978) studied the effect of hexachlorobenzene and acetone on growth and ultra-structure of the fresh algae *Chlorella pyrenoidosa.* They observed many deformed and dented surfaces cells and partly disintegrated cell membranes and cytoplasm in several cells while organelles like chloroplasts and mitochondria did not show obvious changes in ultra-structure.

Table 4.1. Variation in optical density at 750 nm and the 8-d IC_{25} and SC_{20} of *Dunaliella tertiolecta* exposed to DB in the presence and absence of glucose.

Control	% dilution (v/v)				$IC_{25}^{\#}$ % dilution (v/v)	TU*	SC_{20} % dilution (v/v)	TU**
	0	30	60	90				
DYE BATH								
AUC	0.44	0.49	0.28	0.20				
CV	2.83	2.63	14.93	1.06	54.90±2.22##	1.82	24.94±0.37	4.00
Ig (%)		-11.29	34.55	53.15				
DYE BATH with 0.1 g L^{-1} GLUCOSE								
AUC	0.44	0.51	0.61	0.46				
CV	2.83	5.96	0.89	9.88	>100	<1	24.74±1.27 78.72±9.24	4.04 1.27
Ig (%)		-18.75	-38.65	-6.09				
DYE BATH with 0.2 g L^{-1} GLUCOSE								
AUC	0.44	0.56	0.50	0.37				
CV	2.83	6.27	1.414	0.58	>100	<1	12.05±1.80 80.30±7.04	8.29 1.24
Ig (%)		-32.17	-39.41	17.50				

AUC = Mean values from repeated tests (n=3),
CV = Coefficient of variation
Ig (%) = % growth inhibition/stimulation; negative values show stimulation of growth with respect to the control
IC_{25} = 25% Inhibition concentration
SC_{20} = 20% Stimulation concentration
TU = Toxic Unit in terms of IC_{25}
# = 25% Inhibition concentration calculated from the curve fitting analysis
= ± values show standard deviation
* = Toxic Unit in terms of IC_{25}
** = Toxic Unit in terms of SC_{20}

TU value of DB was 1.82 when TU values calculated on the basis of 8-d IC_{25}. Consequently, DB was moderately toxic (TU>1.0, Villegas-Navarro et al., 2001). However, DB with both concentrations of glucose was not toxic with TU<1.0 (Table 4.1). On the other hand, DB was very toxic (TU≥3) when TU values were evaluated on the basis of 8-d SC_{20}. TU values of DB with 0.1 g L^{-1} glucose and 0.2 g L^{-1} glucose were in the range of 1.27-4.04 and 1.24-8.29, respectively. This reflects that DB with 0.1 g L^{-1} and 0.2 g L^{-1} glucose were moderately toxic (TU>1.0) at high dilutions but they were very toxic (TU≥3) at low dilutions.

TU in terms of SC_{20} and IC_{25} indicating that DB may cause adverse effects in an aquatic environment by stimulation of algal growth at low concentrations since the entire aquatic ecosystem may be influenced by changes in algal populations. If the biomass of algae becomes too high or if certain species become abundant, water quality may be negatively impacted. Decreased water transparency and oxygen consumption in bottom waters after settling are two principal consequences of algal overproductivity. Decreases in water transparency may effect growth and survival of higher order, vascular aquatic plants and cause change in fish populations. Additionally, long term effects of continuous low-level exposure to chemicals and their metabolites are not well understood. On the other hand, the presence of DB in aquatic environment at high concentrations may reduce the growth of algae because of its inhibition effect. This can be attributed to the high sensitivity and low tolerance of algae to the high concentration of DB. Additionally, increasing the amount of dye increases solution turbidity and reduces light penetrability. If light saturation is not achieved, then a decrease in the quantal flux in the photosynthetically active spectral region will inevitably result in reduced algal growth, independent from toxicity of the test substance.

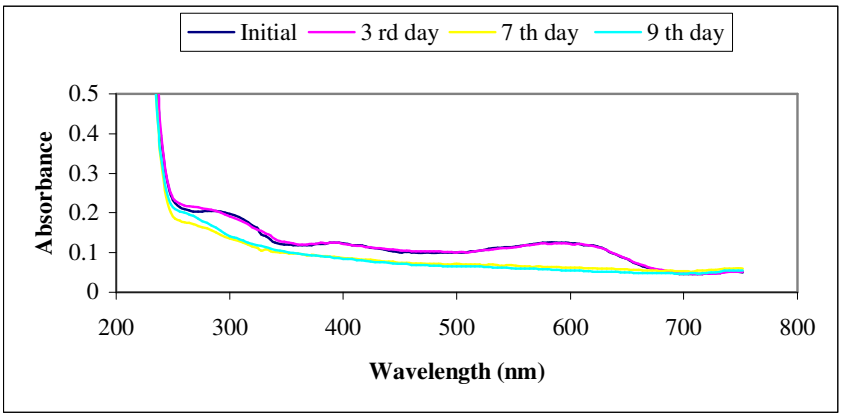
4.2.2. Decolorization of Dye Bath with *Dunaliella tertiolecta* in the Presence and Absence of Glucose

The decolorization of DB with and without glucose was tested for dilutions ranging from 30% (v/v) to 90% (v/v). The cultures were monitored by considering parameters like, change in the absorbance of the prepared dye solutions, decolorization period and the effect of glucose. The UV-Vis spectra of all tested samples collected from the batch culture

during a reaction period of nine days of incubation are shown in Figures from 4.3 to 4.5 to be able to determine absorbance removal at the wavelengths which DB gives maximum absorbance.

Concentration-dependent decolorization was observed. The time overlaid UV-Vis spectra of test samples of DB collected during the decolorization experiments are shown in Figures from 4.3 to 4.5. The intensity of the characteristic absorption peaks of DB at 600 and 288 nm decreased drastically within seven days for 30% and 60% (v/v). *D. tertiolecta* decolorized the DB at low dilutions (30% and 60% dilutions (v/v)) resulting in complete decolorization after seven days of incubation although there was negligible decolorization within the first three days (Figures 4.3 and 4.4 (a)). It can be seen that decolorization was very slow during the initial three days, then, maximum decolorization took place between the third and seventh days. Addition of 0.1 g L^{-1} and 0.2 g L^{-1} glucose did not change the decolorization time of DB for 30% and 60% (v/v) with *D. tertiolecta* (Figures 4.3 and 4.4 (b) and (c), respectively).

Decolorization results of DB with and without glucose obtained with *D. tertiolecta* culture were unsatisfactory for 90% (v/v) (Figure 4.5). Complete decolorization of 90% (v/v) was not obtained with this culture within nine days of incubation. This can be attributed to the high sensitivity and low tolerance of algae to the high concentration of dye which is already expected to become toxic to the microorganisms at higher concentrations. This is supported by the IC_{25} value (54.90% (v/v)) of DB. Toxicity of DB to the algae at high concentration is likely to be the presence of high concentration of multiple azo bonds from six dyes. Paszcynski et al., (1992) reported that in some dyes the low decolorization could be attributed to the complexity of their chromopheres, but the overall complexity alone is not an indicator of the difficulty of decolorization of a particular dye. Even small structural differences can affect the decolorization process. The early step in azo dye decolorization –the breaking of the azo bond- and further degradation involving aromatic cleavage, depends on the identity, number and position of functional groups in the aromatic region and the resulting interaction with the azo bonds (Paszcynski et al., 1992; Spadora et al., 1992; Swamy and Ramsay., 1999).



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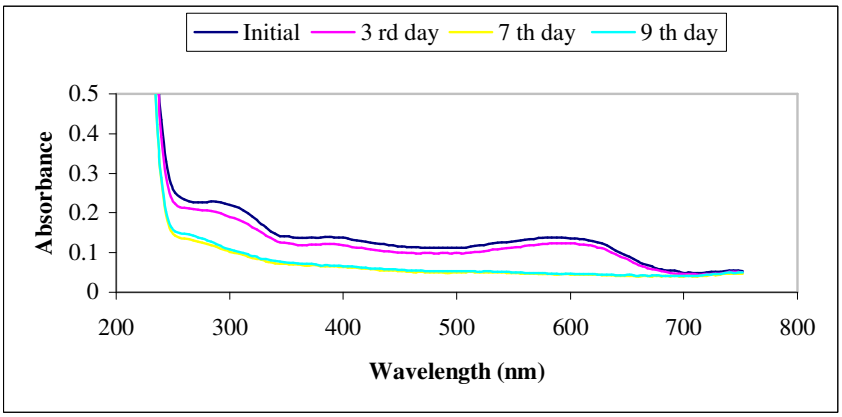
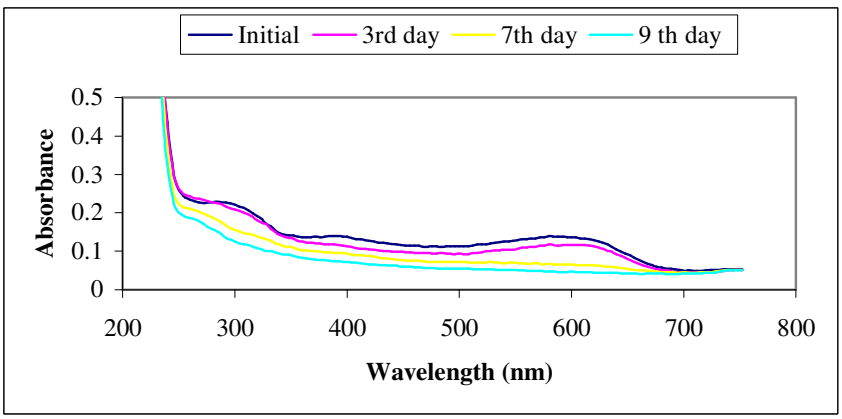
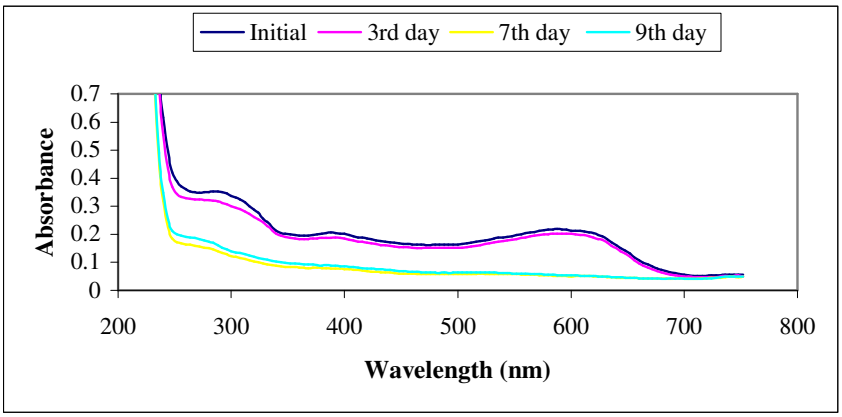


(b)



(c)

Figure 4.3. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 30% dilution (v/v) of a) DB, b) DB and 0.1 g L^{-1} glucose and c) DB and 0.2 g L^{-1} glucose.



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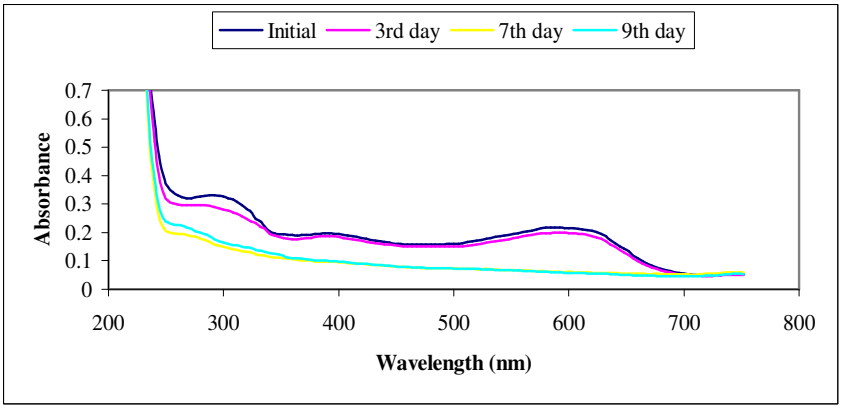

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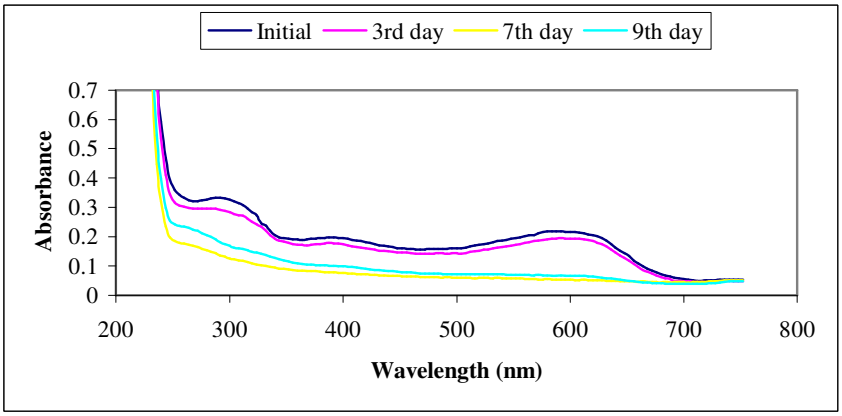
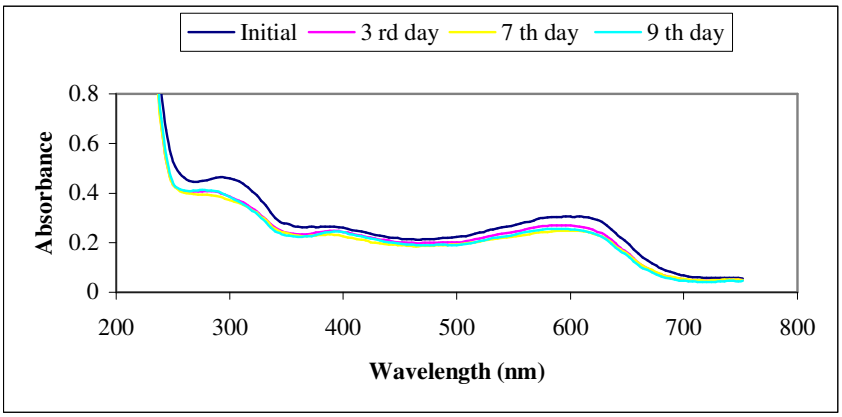
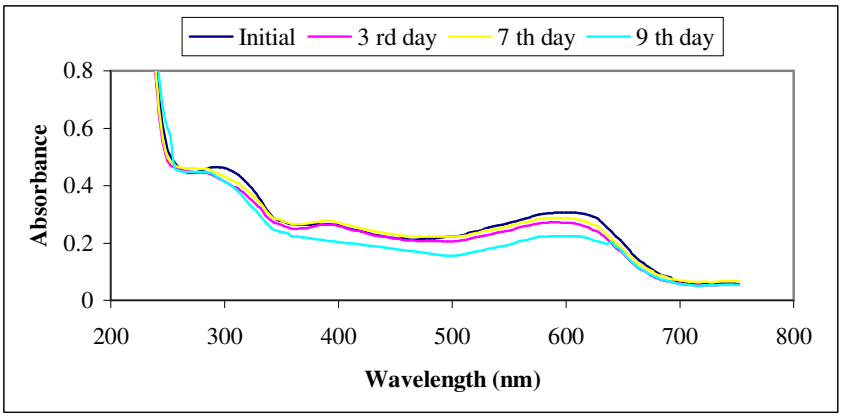


(c)

Figure 4.4. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 60% dilution (v/v) of a) DB, b) DB and 0.1 g L^{-1} glucose and c) DB and 0.2 g L^{-1} glucose.



(a)



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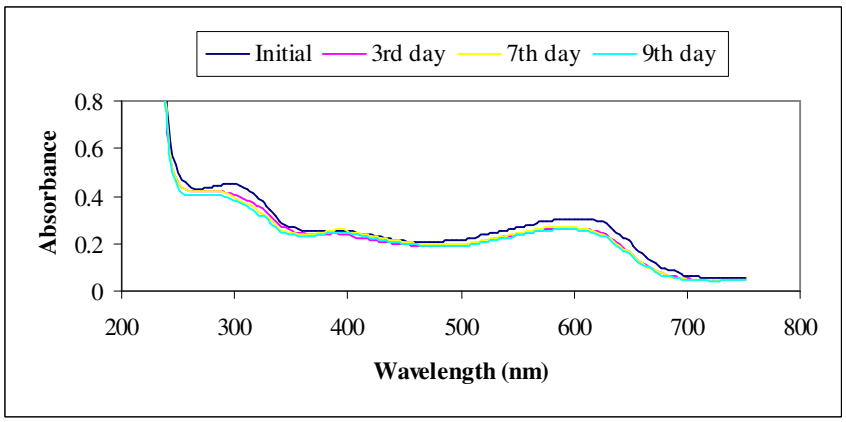


(c)

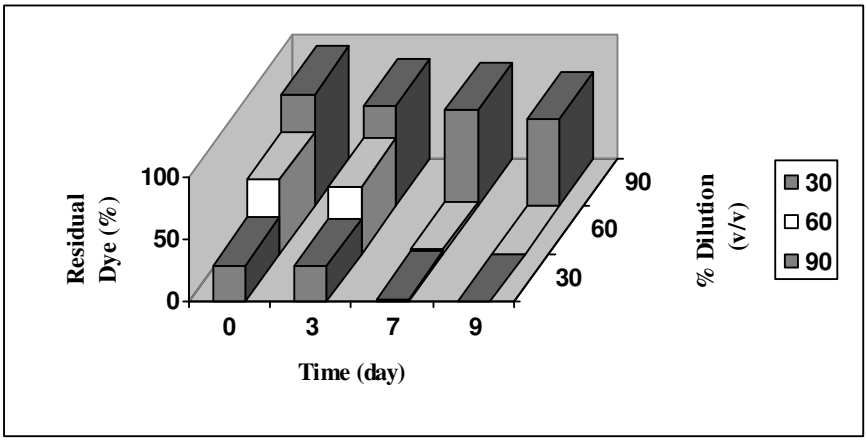
Figure 4.5. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 90% dilution (v/v) of a) DB, b) DB and 0.1 g L^{-1} glucose and c) DB and 0.2 g L^{-1} glucose.

The observations indicated that although low dilutions of DB can be removed without a co-substrate by the algae, the color removal efficiency was 23% for high dilution of DB (90% (v/v)). But we expected to observe that glucose has some beneficial effects such as increasing the decolorization efficiency or decreasing the time period of spectral changes. However, there was no significant difference between decolorization of DB with and without glucose for the studied dilutions. The amount of co-substrate used (0.1 $g.L^{-1}$ and 0.2 g L^{-1} glucose) may not be enough to enhance the decolorization of DB at high dilutions. In addition increasing glucose concentration from 0.1 g L^{-1} to 0.2 g L^{-1} did not effect the decolorization of DB. However, Saçan and Balcıoğlu (2000) reported that the presence of 2 g L^{-1} glucose was clearly beneficial for decolorization of the same DB at high dilutions (>64% (v/v) and color loss reached 40% in glucose containing medium, and only 7% in glucose free medium. Glucose has also been added to enhance the decolorization performance of biological systems in several studies (Haug et al., 1991; Charliell et al., 1995; Kapdan et al., 2000). On the other hand, several scientists reported that glucose inhibited the decolorizing activity (Chang et al., 1978; Knapp and Newby, 1995; White, 1995).

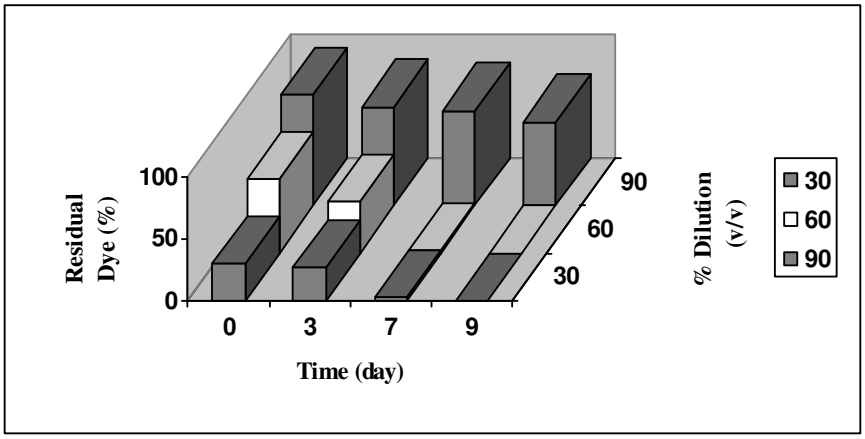
4.2.3. Color Changes during the Decolorization of Dye Bath with and without Glucose

The color values of DB with and without glucose were also tested for dilutions ranging from 30% (v/v) to 90% (v/v) as a platinum cobalt (Pt-Co) unit in order to support the changes in the absorption spectra of DB with *D. tertiolecta*. The variation of dye removal values are depicted in Figure 4.6 for DB with and without glucose for all studied dilutions. The results showed a positive correlation between color and spectral changes.

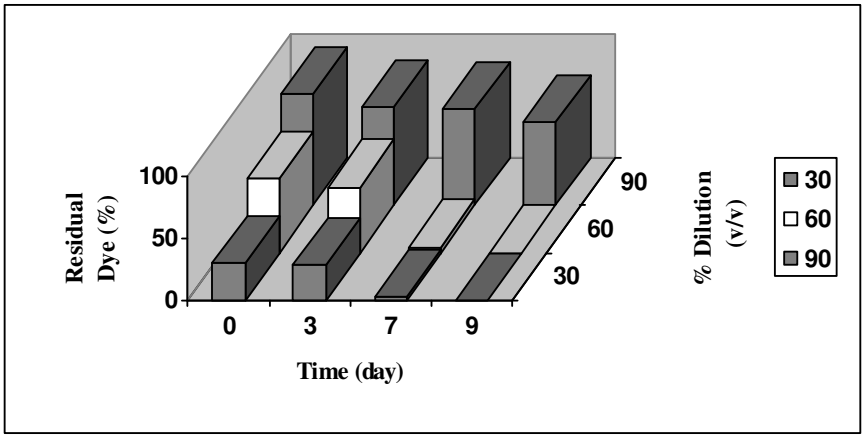
The increase in dye concentration resulted in reduction of the color removal efficiencies. Almost no color removal was observed with *D. tertiolecta* within the first three days at all studied dilutions with and without glucose. The color removal efficiencies were 5%, 10% and 11% for 30%, 60% and 90% (v/v) within three days, respectively. However, color values decreased rather sharply within the next four days resulting in complete decolorization within nine days of incubation for 30% and 60% (v/v). Decolorization result of DB with and without glucose obtained with *D. tertiolecta* culture were unsatisfactory for 90% (v/v) dilutions within nine days of incubation which correspond to only 23%, 25% and 26% color removal efficiency for DB, DB with 0.1 g L^{-1}



(a)



(b)



(c)

Figure 4.6. Color changes in the medium containing DB a) singly b) with 0.1 g L^{-1} glucose c) with 0.1 g L^{-1} glucose.

glucose and DB with 0.2 g L^{-1} glucose, respectively (Figure 4.6).

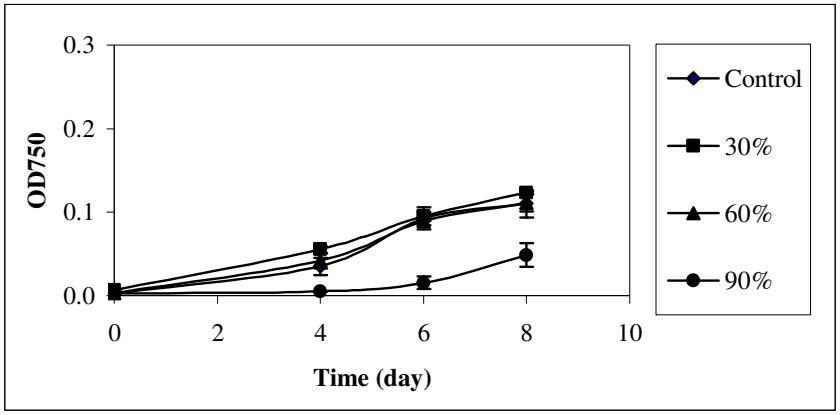
4.2.4. Growth of *Dunaliella tertiolecta* Exposed to Dye Bath with and without Glycerol

To evaluate the effect of toxicity of the DB with and without glycerol on the growth of *Dunaliella tertiolecta* batch studies were conducted with the same dilutions of DB used in glucose experiments. Growth curves of *D. tertiolecta* exposed to different dilution ratios of DB with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol and without glycerol were given in Figure 4.7. Error bars show the mean standard deviation between triplicates. All studied dilutions revealed a significant difference from the control ($p<0.05$).

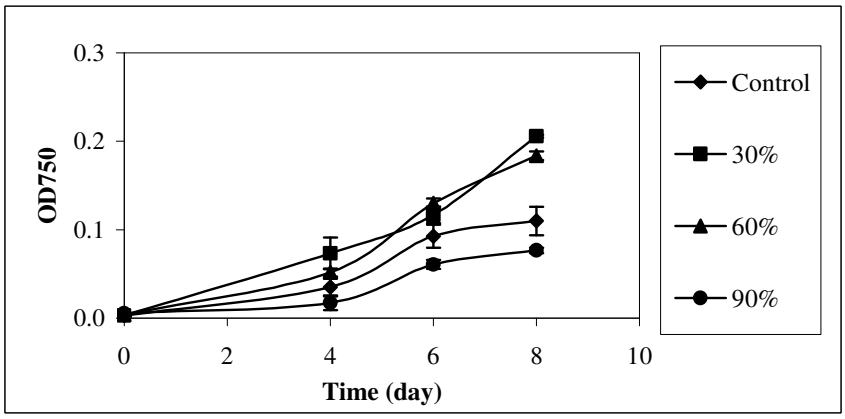
Algal growth was observed during the eight days of incubation. A lag phase duration (four days) was observed to be almost the same at all dilutions tested (30, 60, and 90% dilutions (v/v)). Although in the exponential phase, the growth of algae exposed to DB at all dilutions except 30% (v/v) was lower than that of the control, addition of both 0.1 g L^{-1} and 0.2 g L^{-1} glycerol resulted in stimulation of the growth of algae for 60% (v/v) compared to single exposure of DB (Figure 4.7 and Table 4.2). Hormetic response (low-dose stimulation, high-dose inhibition) was observed in DB with and without glycerol.

Calculated area under the growth curves (AUC) from optical density (OD_{750}) were tabulated in Table 4.2 together with the 8-d IC_{25} and SC_{20} and TU values. The 8-d SC_{20} values observed for DB, DB with 0.1 g L^{-1} glycerol, and DB with 0 2 g L^{-1} glycerol were in the range of 24.94%, 6.89-70.90% and 6.63-71.49% (v/v), respectively. However inhibitory effect was observed at a higher dilution percentage. DB, DB with 0.1 g L^{-1} glycerol and DB with 0.2 g L^{-1} glycerol were toxic to algae with the 8-d IC_{25} values of 54.90±2.22%, 85.08±0.77% and 83.92±1.37% (v/v), respectively. When the 8-d IC_{25} and SC_{20} values of DB were considered, addition of 0.1 g L^{-1} and 0.2 g L^{-1} glycerol as a carbon and energy source increased the stimulatory effect of DB at low dilutions significantly compared to single exposure of DB and the control ($p<0.05$).

TU values of DB, DB with 0.1 g L^{-1} glycerol and DB with 0.2 g L^{-1} glycerol were 1.82, 1.17 and 1.19, respectively, when TU values calculated on the basis of 8-d IC_{25}. Consequently, DB, DB with 0.1 g L^{-1} glycerol and DB with 0.2 g L^{-1} glycerol were



(a)



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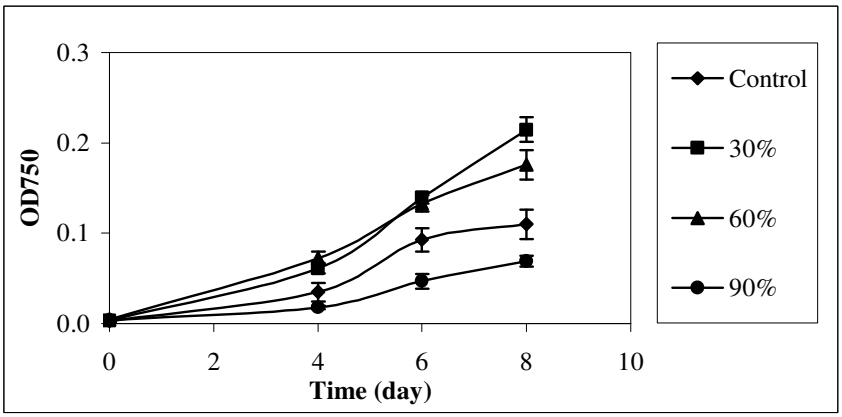


(c)

Figure 4.7. Growth curves of *D. tertiolecta* exposed to a) DB b) DB and 0.1 g L^{-1} glycerol and c) DB and 0.2 g L^{-1} glycerol.

Table 4.2. Variation in optical density at 750 nm and the 8-d IC_{25} and SC_{20} of *Dunaliella tertiolecta* exposed to DB in the presence and absence of glycerol.

Control	% dilution (v/v)				IC_{25} # % dilution (v/v)	TU*	SC_{20} % dilution (v/v)	TU**
	0	30	60	90				
DYE BATH								
AUC	0.44	0.49	0.28	0.20	54.90±2.22##	1.82	24.94±0.37	4.00
CV	2.83	2.63	14.93	1.06				
Ig (%)		-11.29	34.55	52.81				
DYE BATH with 0.1 g L^{-1} GLYCEROL								
AUC	0.44	0.64	0.57	0.21	85.08±0.77	1.17	6.98±0.61 70.90±1.07	14.31 1.41
CV	2.83	4.62	1.23	10.36				
Ig (%)		-57.96	-44.08	44.19				
DYE BATH with 0.2 g L^{-1} GLYCEROL								
AUC	0.44	0.65	0.63	0.19	83.92±1.37	1.19	6.63±0.75 71.49±0.29	15.07 1.39
CV	2.83	5.79	2.27	15.18				
Ig (%)		-59.57	-54.85	54.85				

AUC = Mean values from repeated tests (n=3),
CV = Coefficient of variation
Ig (%) = % growth inhibition/stimulation; negative values show stimulation of growth with respect to the control
IC_{25} = 25% Inhibition concentration
SC_{20} = 20% Stimulation concentration
TU = Toxic Unit in terms of IC_{25}
# = 25% Inhibition concentration calculated from the curve fitting analysis
= ± values show standard deviation
* = Toxic Unit in terms of IC_{25}
** = Toxic Unit in terms of SC_{20}

moderately toxic (TU>1.0, Villegas-Navarro et al., 2001). On the other hand, DB was very toxic (TU≥3) when TU values were evaluated on the basis of 8-d SC_{20}. TU values of DB with 0.1 g L^{-1} glycerol and 0.2 g L^{-1} glycerol were in the range of 1.41-14.31 and 1.39-15.07, respectively. This reflects that DB with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol were moderately toxic (TU>1.0) at high dilutions but they were very toxic (TU≥3) at low dilutions. TU in terms of SC_{20} and IC_{25} indicating that DB may cause adverse effects in an aquatic environment by stimulation of algal growth at low concentrations and an inhibition of algal growth at high concentrations. These adverse effects were discussed in section 4.2.1.

4.2.5. Decolorization of Dye Bath with *Dunaliella tertiolecta* in the Presence and Absence of Glycerol

The decolorization of DB with and without glycerol was tested for dilutions ranging from 30% (v/v) to 90% (v/v). The cultures were monitored by considering parameters like, change in the absorbance of the prepared dye solutions, decolorization period and the effect of glycerol. The UV-Vis spectra of all tested samples collected from the batch culture during a reaction period of eight days of incubation are shown in Figures from 4.8 to 4.10 to be able to determine absorbance removal at the wavelengths which DB gives maximum absorbance.

Concentration-dependent decolorization was observed. The time overlaid UV-Vis spectra of test samples of DB collected during the decolorization experiments are shown in Figures from 4.8 to 4.10. The intensity of the characteristic absorption peaks of DB at 600 and 288 nm decreased drastically within six days for 30% and 60% (v/v). *D. tertiolecta* decolorized the DB at low dilutions (30% and 60% dilutions (v/v)) resulting in complete decolorization during six days of incubation although there was negligible decolorization within the first four days (Figures 4.8 and 4.9 (a)). However, when the batch culture was supplemented with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol, the intensity of characteristic adsorption peaks of DB at 600 and 288 nm decreased drastically within four days for 30% (v/v) (Figures 4.8 (b) and (c), respectively). While addition of 0.1 g L^{-1} glycerol caused more decrease in maximum peaks of DB for 60% (v/v), decolorization time of DB for 60%

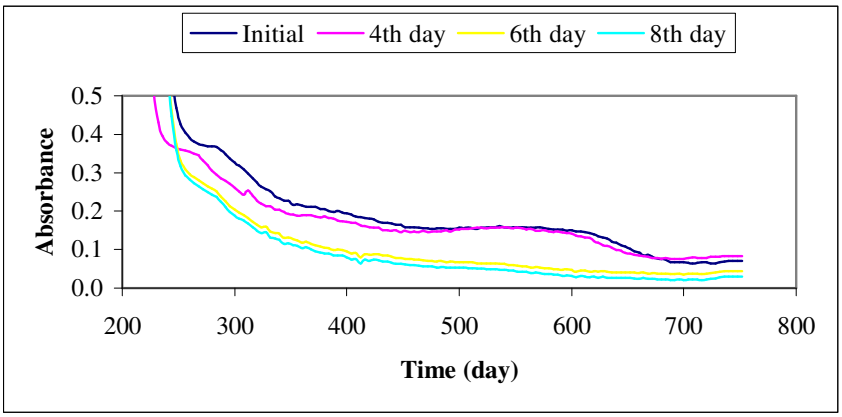
(v/v) decreased from six days to four days with the addition of 0.2 g L^{-1} glycerol (Figures 4.9 (b) and (c), respectively) .

Decolorization results of DB with and without glycerol obtained with *D. tertiolecta* culture was unsatisfactory for 90% (v/v) (Figure 4.10 (a), (b) and (c), respectively). Complete decolorization of 90% (v/v) was not obtained with this culture within eight days of incubation. This can be attributed to the high sensitivity and low tolerance of algae to the high concentration of dye which is already expected to become toxic to the microorganisms at higher concentrations. This is supported by the 8-d IC_{25} value (54.90% (v/v)) of DB. Toxicity of DB to the algae at high concentration is likely to be the presence of high concentration of multiple azo bonds from six dyes as reported by Paszcynski et al., (1992) and stated in section 4.2.2.

Although addition of both concentration of glycerol stimulated the growth of algae at all studied dilutions, decolorization of DB for 90% (v/v) with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol didn't change. However, addition of both 0.1 g L^{-1} and 0.2 g L^{-1} glycerol has some beneficial effects such as showing more decolorization efficiency for 30% (v/v) at the fourth day and decreasing the time period of spectral changes from six days to four days for 60% (v/v) when the batch culture was supplemented with 0.2 g L^{-1} glycerol. This result showed that *D. tertiolecta* can use dye stuff (DB) as a carbon source but an external carbon source addition is necessary to speed up the decolorization. There is no study in the literature, which use glycerol as a co-substrate for decolorization of textile dyes. Glycerol as an additional carbon and energy source on the decolorization of DB by *D. tertiolecta* has not been studied yet and its decolorization abilities could be promising for further biotechnological applications. However, further decolorization tests are required to support these findings, by changing the dosage of glycerol.

4.2.6. Color Changes during the Decolorization of Dye Bath with and without Glycerol

The color values of DB with and without glycerol was also tested for dilutions ranging from 30% (v/v) to 90% (v/v) as a platinum cobalt (Pt-Co) unit in order to support the changes in the absorption spectra of DB with *D. tertiolecta*. The variation of dye



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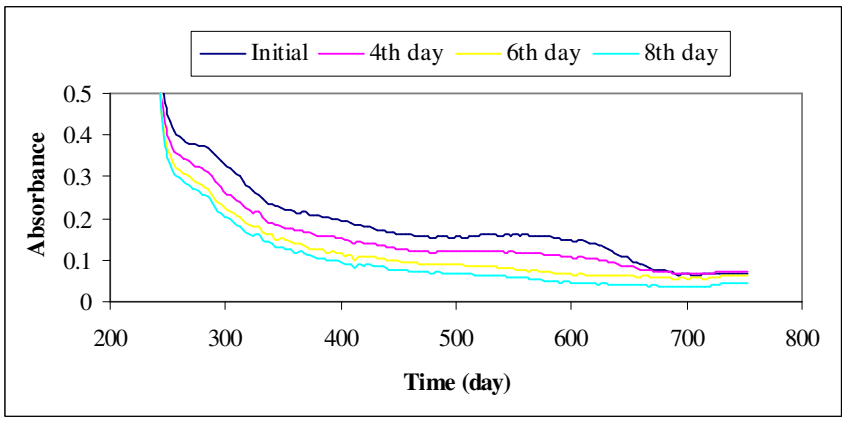

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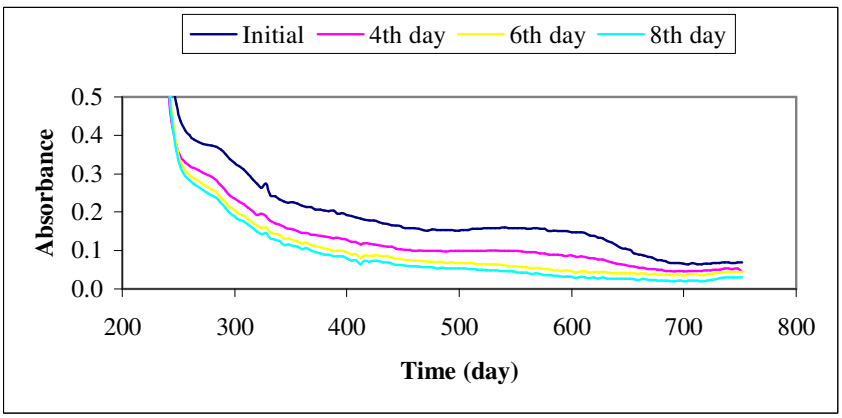
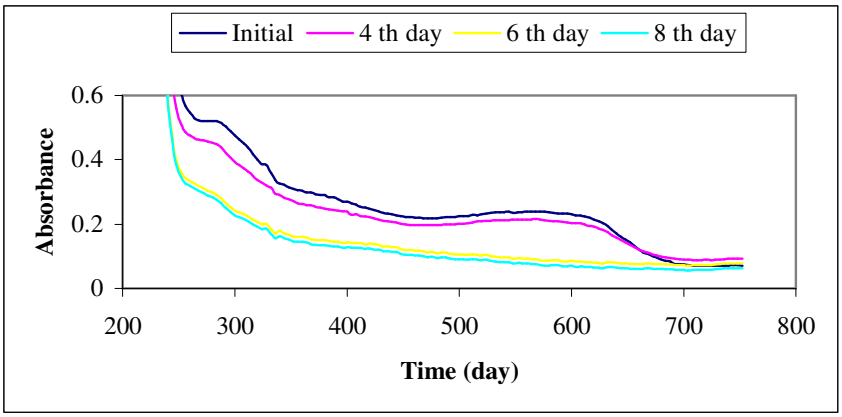
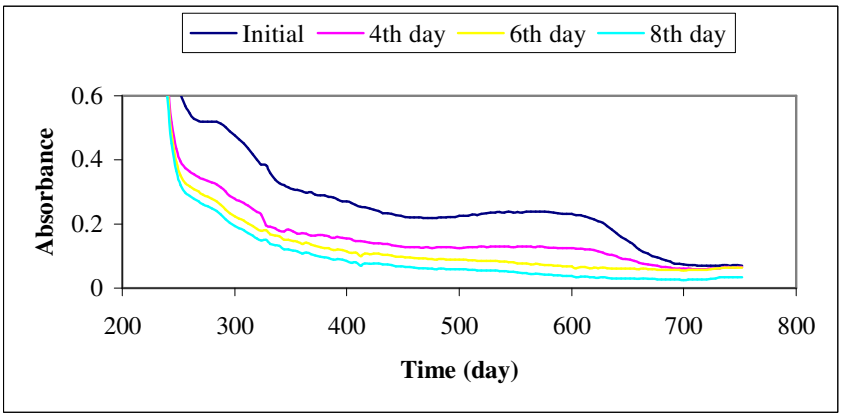


(c)

Figure 4.8. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 30% c (v/v) of a) DB, b) DB and 0.1 g L^{-1} glycerol and c) DB and 0.2 g L^{-1} glycerol.



(a)



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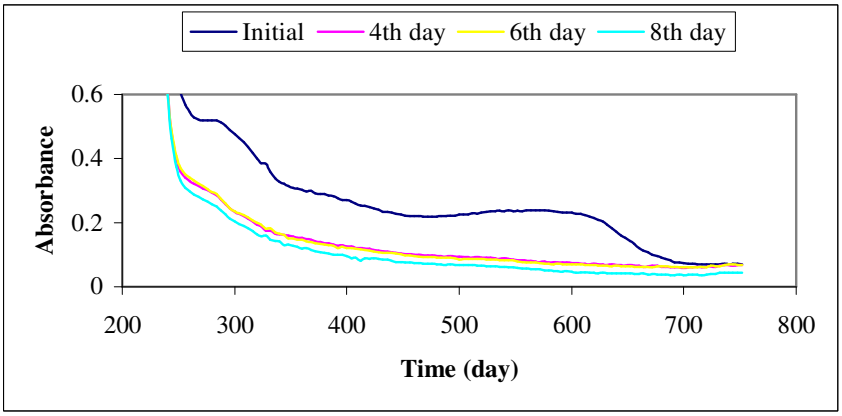
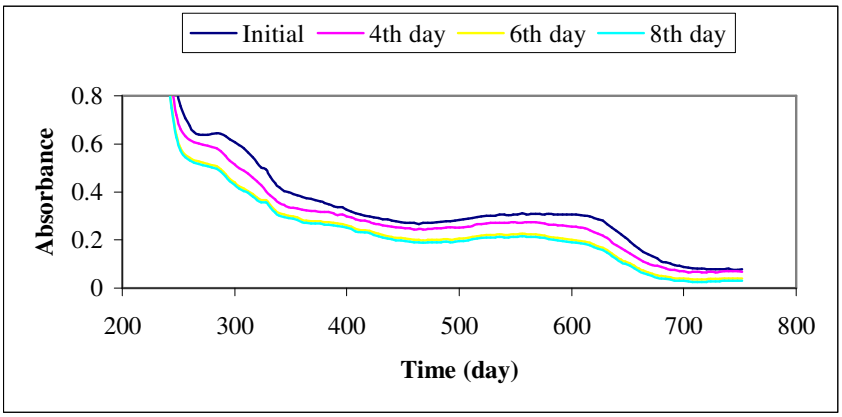
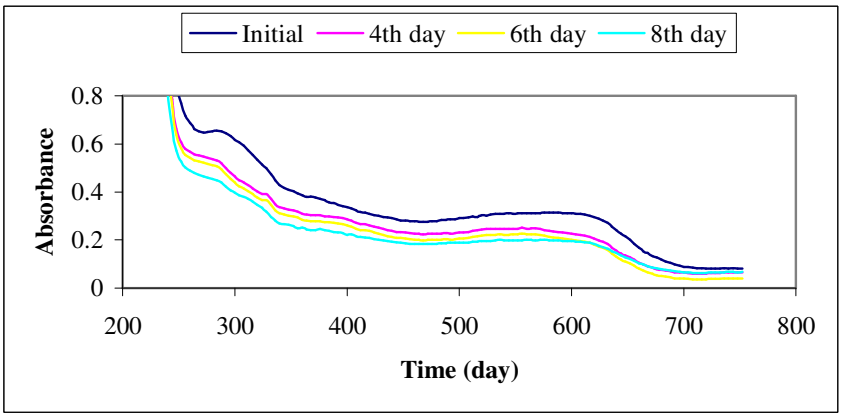


(c)

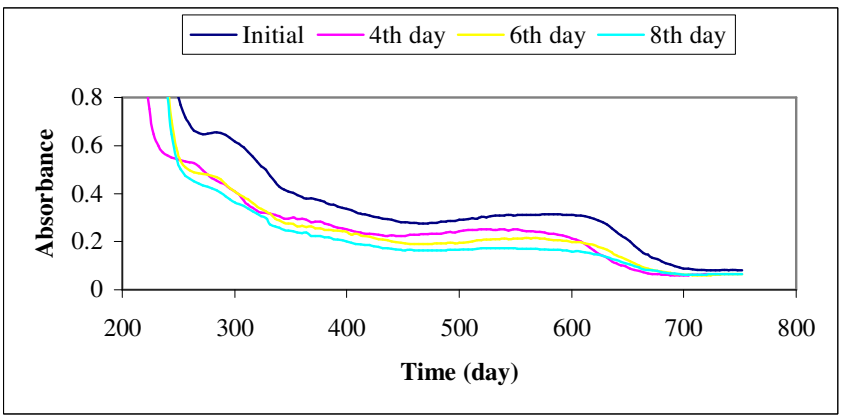
Figure 4.9. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 60% dilution (v/v) of a) DB, b) DB and 0.1 g L^{-1} glycerol and c) DB and 0.2 g L^{-1} glycerol.



(a)



(b)



(c)

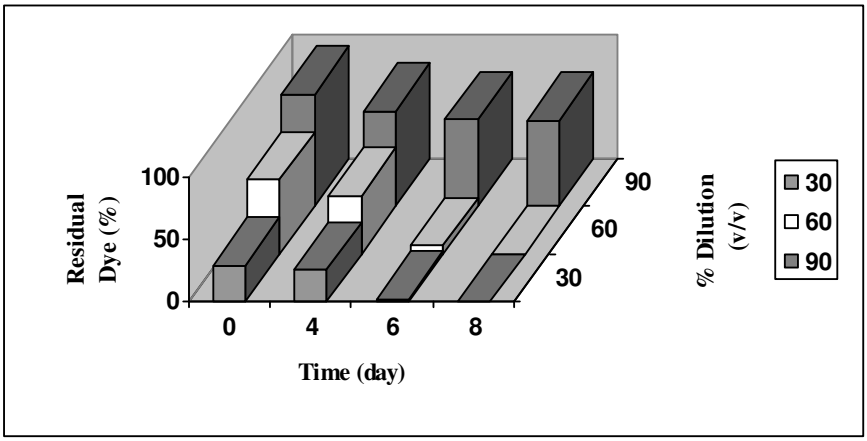
Figure 4.10. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 90% dilution (v/v) of a) DB, b) DB and 0.1 g L^{-1} glycerol and c) DB and 0.2 g L^{-1} glycerol.

removal values are depicted in Figure 4.11 for DB with and without glycerol for all studied dilutions. The results showed a positive correlation between color and spectral changes.

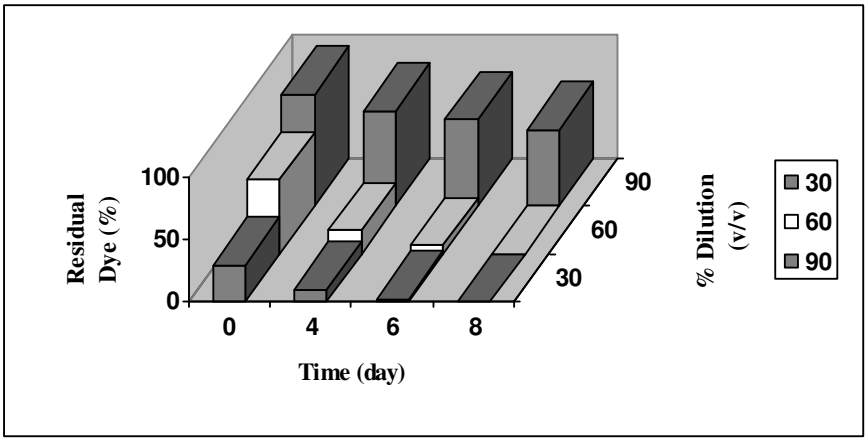
The increase in dye concentration resulted in reduction of the color removal efficiencies. Almost no color removal was observed with *D. tertiolecta* within the first four days at all studied dilutions. The color removal efficiencies were 14%, 22% and 15 % for 30%, 60% and 90% (v/v) of DB within two days, respectively. Then, color values decreased rather sharply within the next four days resulting in complete decolorization during six days of incubation for 30% and 60% (v/v). However, when batch culture was supplemented with 0.1 g L^{-1} glycerol, 65% and 69% color removal was accomplished for 30% and 60% (v/v) of DB within four days. Complete color removal was obtained with *D. tertiolecta* for 30% and 60% (v/v) of DB with 0.1 g L^{-1} glycerol during eight days of incubation. Additionally, when the batch culture was supplemented with 0.2 g L^{-1} glycerol, color values decreased rather sharply within the first four days resulting in 84% and 87% color removal efficiencies for 30% and 60% (v/v) (Figure 4.11). Decolorization result of DB with and without glycerol obtained with *D. tertiolecta* culture were unsatisfactory for 90% (v/v) during eight days of incubation which corresponds to only 25%, 28% and 29% color removal efficiency for DB, DB with 0.1 g L^{-1} glycerol and DB with 0.2 g L^{-1} glycerol, respectively.

4.2.7. pH Changes during Decolorization of Dye Bath with and without Glucose and Glycerol

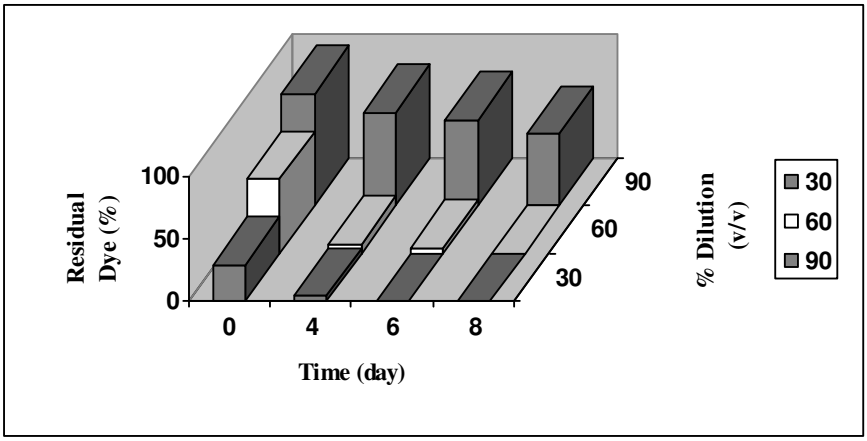
Changes in the pH value of the culture media during the growth period of algae exposed to DB with and without glucose and glycerol are shown in Figures 4.12 and 4.13, respectively. Error bars show the mean standard deviation between triplicates. Range of pH differed notably between control and samples containing DB with and without glucose and glycerol. The average pH change in blank samples was 0.33, 0.98, 1.51 and 1.80 unit for control and 30%, 60% and 90% (v/v), respectively. The pH of all the blanks decreased during eight days because of the dissolution of CO_2 and subsequent formation of H_2CO_3. Algal growth caused marked changes in the pH of the culture media, raising the low pH values (e.g., control and 30% (v/v), Figures 4.12 and 4.13 (a) and (b), respectively) and first lowering and then raising the high ones (e.g., 60 and 90% dilutions (v/v), Figures 4.12



(a)



(b)



(c)

Figure 4.11. Color changes in the medium containing DB a) singly b) with 0.1 g L^{-1} glycerol c) with 0.2 g L^{-1} glycerol

and 4.13 (c) and (d), respectively. In fact, pH increases due to utilization of carbon dioxide and bicarbonate in photosynthesis and growth.

Within nine days all cultures exposed to DB and glucose reached the pH range of 8.14-11.10. A sharp increase was observed in pH from 8.16 to 9.50 for the control and from 9.52 to 9.80 for DB with and without glucose for 30% (v/v), then followed by almost a plateau. On the other hand, pH decreased in the test vessels containing 60% and 90% (v/v) during the first three days of growth, but it rose afterwards. While average 0.72 and 1.48 pH unit decreases were observed in the pH of the samples for 60% and 90% (v/v) with and without glucose, respectively, the average increase in pH for the same dilutions was 0.90 and 1.03 unit, respectively.

The same increasing and decreasing trend was also observed for the pH of the flasks containing DB with and without glycerol for the control and all dilutions (30%, 60% and 90% (v/v)). Within eight days all cultures reached the pH range of 8.48-10.90. A sharp increase was observed in pH from 8.78 to 9.84 for control and from 9.17 to 10.01 for DB with and without glycerol for 30% (v/v), then followed by almost a plateau. On the other hand, pH decreased in the test vessels containing 60% and 90% (v/v) during the first four days of growth, but it rose afterwards. While average 0.59 and 1.07 pH unit decreases were observed in the pH of the samples for 60% and 90% (v/v) with and without glycerol respectively, the average increase in pH for the same dilutions was 0.46 and 1.00 unit, respectively.

Glucose and glycerol did not change the initial pH and pH changing trend of the medium containing DB, significantly.

The increase in the pH of the samples for 30%, 60% and 90% (v/v) might be due to the new organic matters which are the end products of degradation of DB. The increase and decrease of the aqueous phase pH values during the dye removal by *D. tertiolecta* may be attributed to the surface interaction between the cell surface and the dye molecule. Algal surface is naturally formed by various chemical groups such as hydroxyl, carboxylate amino and phosphate which are believed to be responsible for the sequestration of

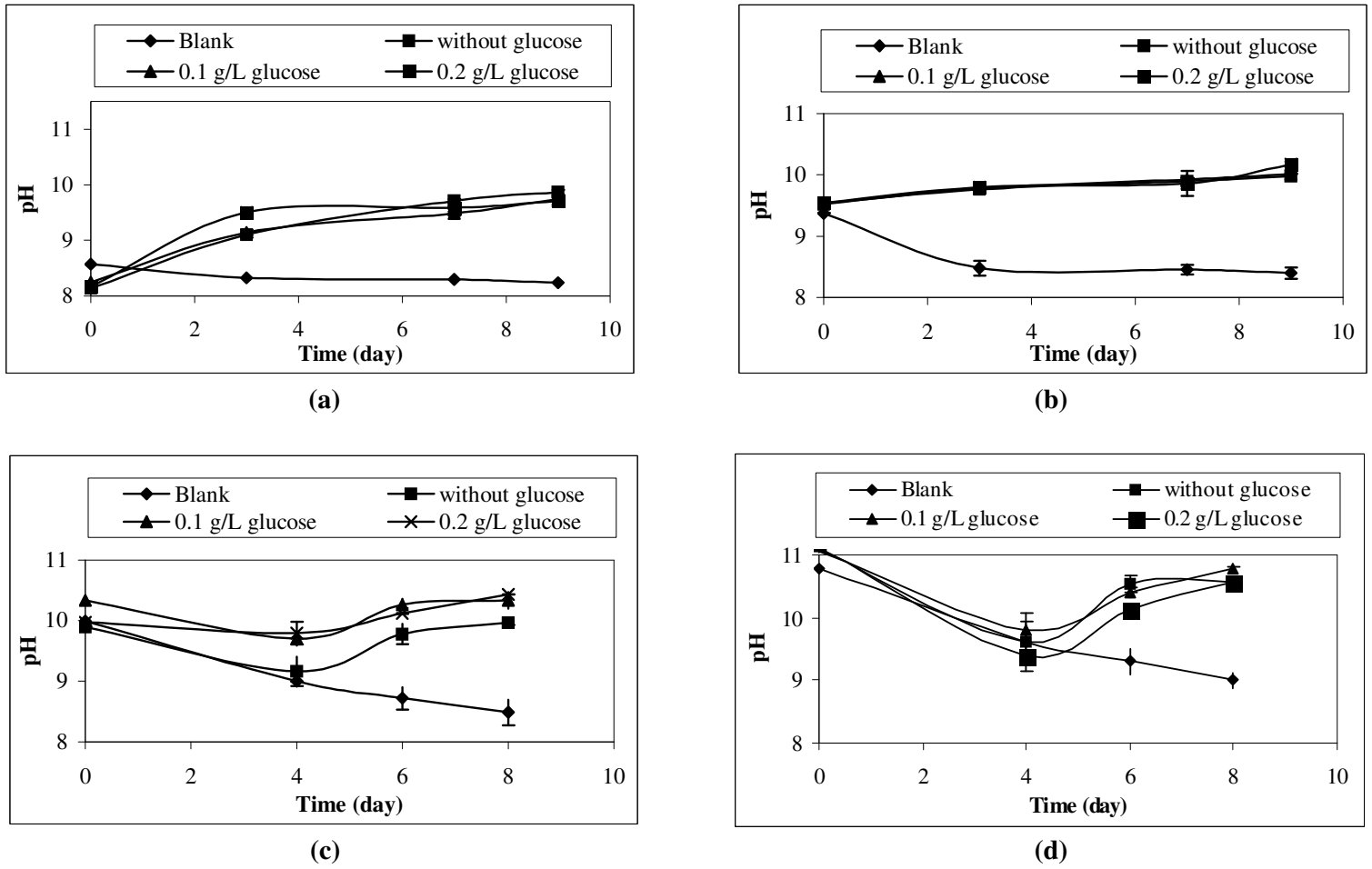


Figure 4.12. pH changes in the medium containing a) Control b) 30% DB, c) 60% DB and d) 90% DB with and without glucose.

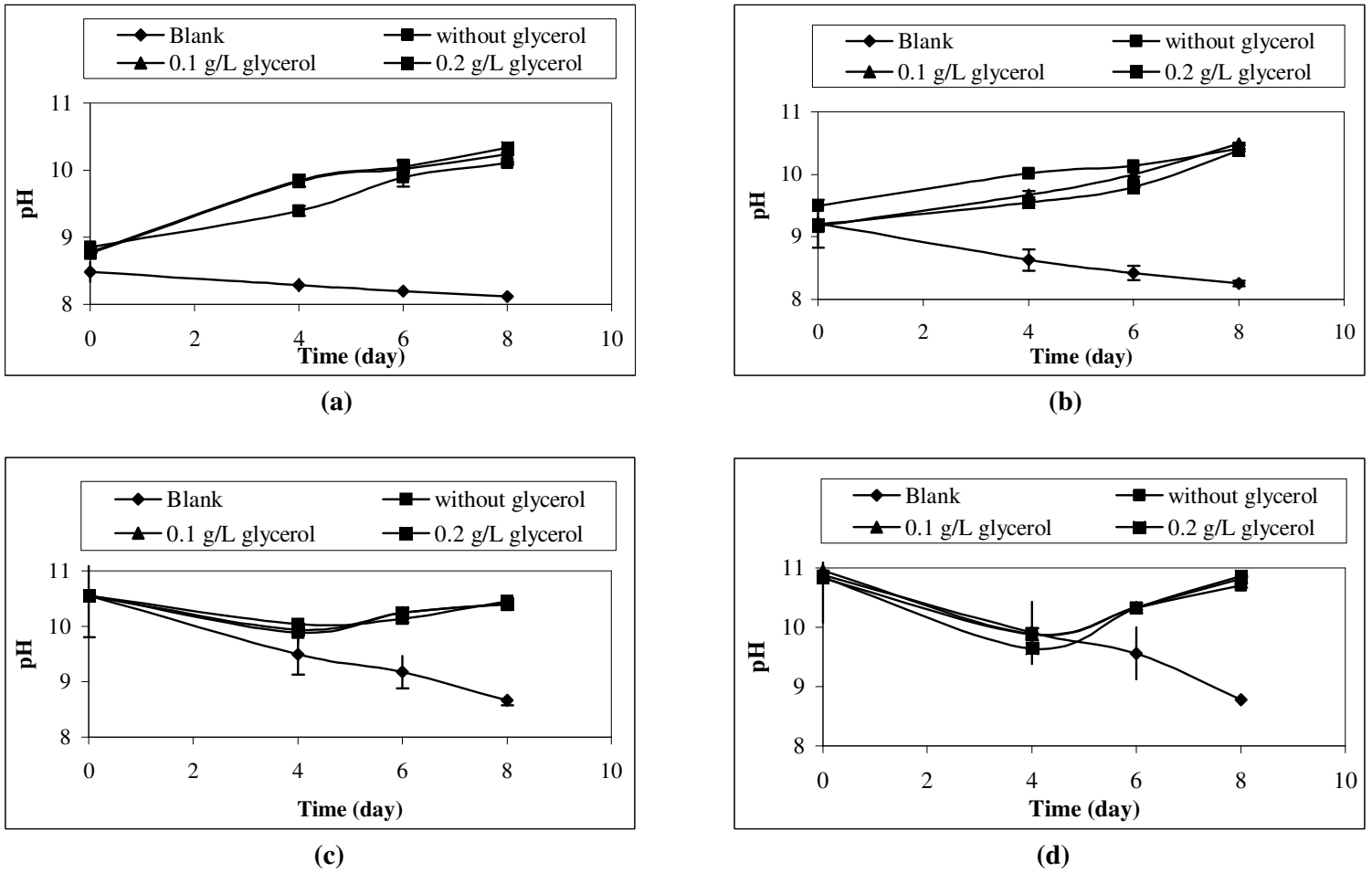


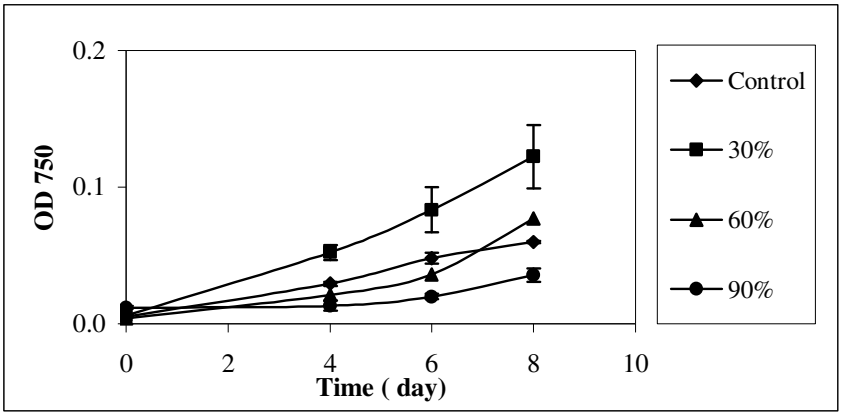
Figure 4.13. pH changes in the medium containing a) Control b) 30% DB, c) 60% DB and d) 90% DB with and without glycerol.

unwanted materials from effluents by two distinct processes: (i) bioaccumulation and (ii) biosorption (Özer et al., 2006). The term biosorption implies a direct interaction between the biosorbent and the dye adsorbate. Algae have been found to be potential suitable biosorbents because of their cheap availability both in fresh or saltwater, relatively high surface area and high binding affinity (Tien et al., 2002). Biosorption on algae may mainly been attributed to the cell wall properties where both electrostatic attraction and complexation play a role (Şatiroğlu et al., 2002). Several investigators observed that maximum adsorption occurs at the isoelectronic point. At lower pH below the isoelectronic point, the surface of algae may acquire a positive charge leading to an increased unionic dye (direct, acidic, reactive) uptake due to the electrostatic force of attraction. Stum and Morgan (1981) reported that the decrease in pH might be due to the low net (zero) surface electric charge and the probability of the contact. Mohan et al., (2002) reported that the increase in pH might be related to the accumulation of dyes by the green algae *Spirogya* cells at pH 7-8. Additionally, alkalization of the growth medium during algal growth may have affected enzyme production (Weich and Graneli, 1989), hence, decolorization efficiency may increased by these enzyme production.

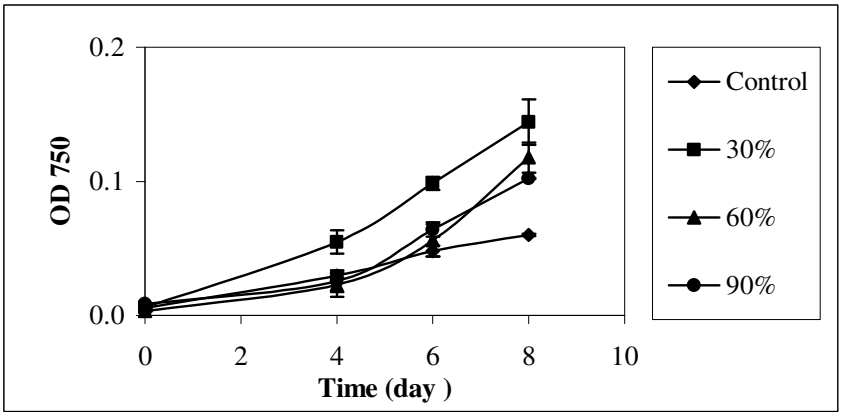
4.3. Reactive Black 5

4.3.1. Growth of *Dunaliella tertiolecta* Exposed to Reactive Black 5 with and without Glucose

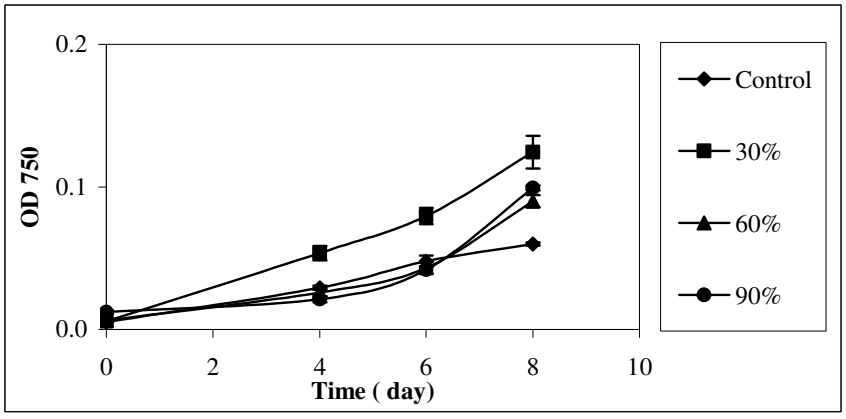
Reactive Black 5 (RB-5) with a sulphatoethylsulphonate reactive group has the highest concentration among the six azo dyes in the dye-bath recipe. To evaluate the effect of toxicity of RB-5 with and without glucose on the growth of *Dunaliella tertiolecta* batch studies were conducted with the same dilutions of DB used in glucose experiments. Growth curves of *D. tertiolecta* exposed to different dilution ratios of RB-5 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose and without glucose were given in Figure 4.14. Error bars show the mean standard deviation between triplicates. All studied dilutions (% (v/v)) revealed a significant difference from the control ($p<0.05$).



(a)



(b)



(c)

Figure 4.14. Growth curves of *D. tertiolecta* exposed to a) RB-5 b) RB-5 and 0.1 g L^{-1} glucose and c) RB-5 and 0.2 g L^{-1} glucose.

Algal growth was observed during the eight days of incubation. The response of algae exposed to RB-5 was similar to that of DB. Algal growth was promoted at low dilutions of the RB-5 and the biomass increased with the increase in number of days. A lag phase duration (four days) was observed to be almost the same at all dilutions tested (30, 60, and 90% dilutions (v/v)). In the exponential phase, the growth of algae exposed to RB-5 at all dilutions except 30% (v/v) was lower than that of the control. However, addition of both 0.1 g L^{-1} and 0.2 g L^{-1} glucose resulted in stimulation of the growth of algae for all dilutions compared to the single exposure of RB-5 and the control (Figure 4.14 and Table 4.3). The growth of algae showed a decreasing trend with increasing dye concentration. Reduction in cell growth may result from the toxicity of dyes to algae through the inhibition of metabolic activities as stated in section 4.2.1.

While hormetic response (low-dose stimulation and high-dose inhibition) was observed in RB-5, only stimulation was observed for RB-5 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose at all dilutions. In other words, glucose eliminated the inhibitory effects of RB-5 on the growth response of algae for all dilutions.

Calculated area under the growth curves (AUC) from optical density (OD_{750}) were tabulated in Table 4.3 together with the 8-d IC_{25} and SC_{20} and TU values. The 8-d SC_{20} values for RB-5, RB-5 with 0.1 g L^{-1} glucose and RB-5 with 0.2 g L^{-1} glucose were in the range of 8.66-51.33%, 4.00-61.00% and 5.33-56.00% (v/v), respectively. However, inhibitory effect was observed at a higher dilution percentage when algae exposed to only RB-5. RB-5 was toxic to algae with the 8-d IC_{25} value of 62.58±6.76% (v/v). Since both concentrations of glucose eliminated the inhibitory effect of RB-5 for the studied dilutions, the 8-d IC_{25} values couldn't be calculated for RB-5 in the presence of glucose. In other words, no toxicity of RB-5 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose was detectable with an IC_{25} of >100%. When the 8-d IC_{25} and SC_{20} values of RB-5 were considered, addition of 0.1 g L^{-1} and 0.2 g L^{-1} glucose as a carbon and energy source increased the stimulatory effect of RB-5 significantly compared to the single exposure of RB-5 and control ($p<0.05$) (Table 4.3).

When TU values calculated on the basis of 8-d IC_{25}, TU value of RB-5 was 1.59. Consequently, RB-5 was moderately toxic (TU>1.0, Villegas-Navarro et al., 2001).

Table 4.3. Variation in optical density at 750 nm and the 8-d IC_{25} and SC_{20} of *Dunaliella tertiolecta* exposed to RB-5 in the presence and absence of glucose.

Control	% dilution (v/v)				IC_{25} % dilution (v/v)	TU*	SC_{20} % dilution (v/v)	TU**
	0	30	60	90				
REACTIVE BLACK 5								
AUC	0.21	0.40	0.18	0.04	62.58#±6.76##	1.59	8.66±1.15 51.33±1.33	11.53 1.94
CV	7.59	15.61	8.36	31.09				
Ig (%)		-92.45	12.26	79.40				
REACTIVE BLACK 5 with 0.1 g L^{-1} GLUCOSE								
AUC	0.21	0.44	0.29	0.23	>100	<1	4.00±1.41 61.00±1.63	25.00 1.63
CV	7.59	0.64	1.71	13.45				
Ig (%)		-108.49	-36.50	-7.07				
REACTIVE BLACK 5 with 0.2 g L^{-1} GLUCOSE								
AUC	0.21	0.42	0.24	0.22	>100	<1	5.33±0.57 56.00±1.73	18.75 1.78
CV	7.59	6.79	0.69	9.83				
Ig (%)		-101.17	-13.20	-3.77				

AUC = Mean values from repeated tests (n=3),
CV = Coefficient of variation
Ig (%) = % growth inhibition/stimulation; negative values show stimulation of growth with respect to the control
IC_{25} = 25% Inhibition concentration
SC_{20} = 20% Stimulation concentration
TU = Toxic Unit in terms of IC_{25}
# = 25% Inhibition concentration calculated from ICp method and the rest was calculated from the curve fitting analysis
= ± values show standard deviation
* = Toxic Unit in terms of IC_{25}
** = Toxic Unit in terms of SC_{20}

However, RB-5 with 0.1 g L^{-1} and 0.2 glucose g L^{-1} was not toxic with TU<1.0 (Table 4.2). On the other hand, TU values of RB-5, RB-5 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose were in the range of 1.94-11.53, 1.63-25.00 and 1.78-21.42, respectively when TU values were evaluated on the basis of 8-d SC_{20}. This reflects that RB-5 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose and without glucose were moderately toxic (TU>1.0) at high dilutions but they were very toxic (TU≥3) at low dilutions. TU in terms of SC_{20} and IC_{25} indicating that RB-5 may cause adverse effects in an aquatic environment by stimulation of algal growth at low concentrations and an inhibition of algal growth at high concentrations as stated in Section 4.2.1. RB-5 with 0.1 g L^{-1} and 0.2 g L^{-1}glucose cause only stimulatory effect on the growth of algae.

4.3.2. Decolorization of Reactive Black 5 with *Dunaliella tertiolecta* in the Presence and Absence of Glucose

The decolorization of RB-5 with and without glucose was tested for dilutions ranging from 30% (v/v) to 90% (v/v). The cultures were monitored by considering parameters like, change in the absorbance of the prepared dye solutions, decolorization period and the effect of glucose. The UV-Vis spectra of all tested samples collected from the batch culture during a reaction period of eight days of incubation are shown in Figures from 4.15 to 4.17 to be able to determine absorbance removal at the wavelengths which RB-5 gives maximum absorbance.

Concentration-dependent decolorization was observed similar to DB. The time overlaid UV-Vis spectra of test samples of RB-5 collected during the decolorization experiments are shown in Figures from 4.15 to 4.17. Initially RB-5 presents two main absorption peaks, one in visible region (600 nm) and another one in UV region (316 nm), which can be ascribed to the presence of chromophoric azo bonds and both aryl and naphthalene-like moieties (Silverstein et al., 1991), respectively. The intensity of the characteristic absorption peaks of RB-5 at 600 and 316 nm decreased drastically within six days for 30 and 60% (v/v). *D. tertiolecta* decolorized the RB-5 at low dilutions (30% and 60% (v/v)) resulting in complete decolorization after six days of incubation although there was approximately 30-40% decrease in absorbance at maximum wavelengths at 600 and 316 nm for 30% and 60% (v/v) within the first four days (Figures 4.15 and 4.16 (a)).

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** = Toxic Unit in terms of SC_{20}

However, RB-5 with 0.1 g L^{-1} and 0.2 glucose g L^{-1} was not toxic with TU<1.0 (Table 4.2). On the other hand, TU values of RB-5, RB-5 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose were in the range of 1.94-11.53, 1.63-25.00 and 1.78-21.42, respectively when TU values were evaluated on the basis of 8-d SC_{20}. This reflects that RB-5 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose and without glucose were moderately toxic (TU>1.0) at high dilutions but they were very toxic (TU≥3) at low dilutions. TU in terms of SC_{20} and IC_{25} indicating that RB-5 may cause adverse effects in an aquatic environment by stimulation of algal growth at low concentrations and an inhibition of algal growth at high concentrations as stated in Section 4.2.1. RB-5 with 0.1 g L^{-1} and 0.2 g L^{-1}glucose cause only stimulatory effect on the growth of algae.

4.3.2. Decolorization of Reactive Black 5 with *Dunaliella tertiolecta* in the Presence and Absence of Glucose

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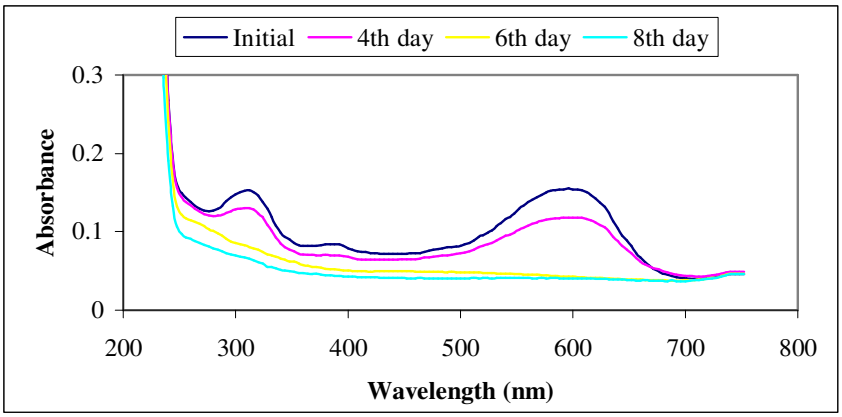
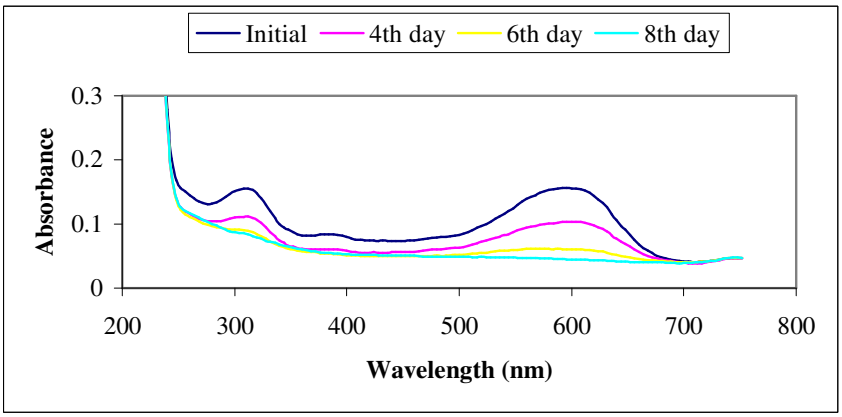
Addition of 0.1 g L^{-1} glucose did not change the decolorization time of RB-5 for 30% and 60% (v/v) dilutions with *D. tertiolecta* (Figures 4.15 and 4.16 (b)). However, when the batch culture was supplemented with 0.2 g L^{-1} glucose, these peaks disappeared completely during four days for 30% (v/v) (Figures 4.15 (c)). It can be seen that decolorization was very slow during initial four days, then maximum decolorization takes place between the fourth and sixth days for 30% and 60% (v/v) with and without glucose except for RB-5 with 0.2 g L^{-1} glucose for 30% (v/v). Approximately 25-33% decrease in treatment time upon addition of 2 g L^{-1} glucose reported in a study carried out by Mohanty et al., (2006) in which they investigated microbial decolorization of RB-5 in a two stage anaerobic–aerobic reactor using acclimatized sludge.

Decolorization result of RB-5 with and without glucose obtained with *D. tertiolecta* culture was unsatisfactory for 90% (v/v) (Figures 4.17). Although approximately 40-50% decrease in absorbance at maximum wavelengths at 600 and 316 nm for 90 % of RB-5 with and without glucose was observed, complete decolorization was not obtained with this culture within eight days of incubation. This can be attributed to the high sensitivity and low tolerance of algae to the high concentration of dye which is already expected to become toxic to the microorganisms at higher concentrations. This is supported by the 8-d IC_{25} value (62.58% (v/v)) of RB-5. Toxicity of RB-5 to the algae at high concentration is likely to be the presence of high concentration of azo bond as reported by Paszcynski et al., (1992) and stated in Section 4.2.2.

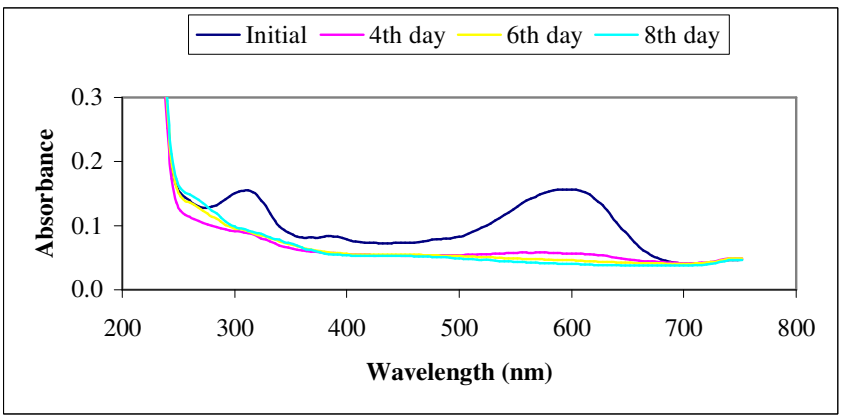
Mohanty et al., (2006) also investigated the decolorization of RB-5 in a two-stage anaerobic-aerobic reactor using a mixed culture of bacteria isolated from textile dye effluent. The results showed that major decolorization was achieved during the anaerobic process. It was possible to achieve more than 90% decolorization of RB-5 (500-2000 mg L^{-1}) and approximately 46% reduction in amine metabolite concentration through two-stage anaerobic-aerobic treatment after a reaction period of two days. They also reported that biodegradation of RB-5 may be expected to comprise two main steps: the reductive cleavage of the azo bond under anaerobic condition and the subsequent aerobic mineralization of metabolites formed in anaerobic process. Cleavege of the azo bond (N=N-) in RB-5 molecule is expected to result in permanent decolorizaiton of the RB-5 dye



(a)

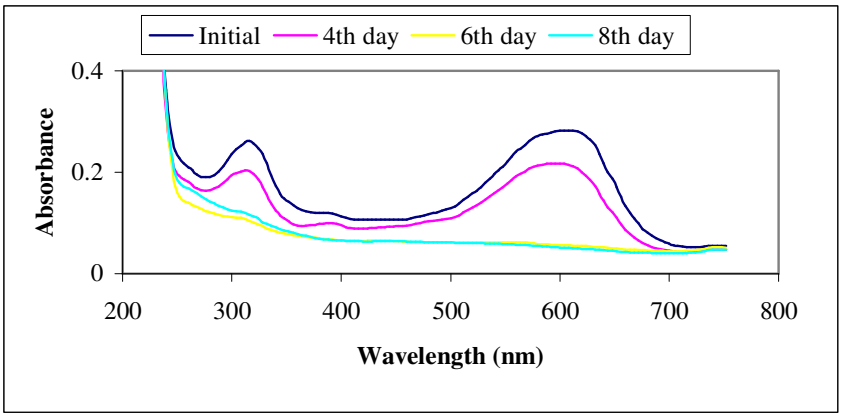


(b)

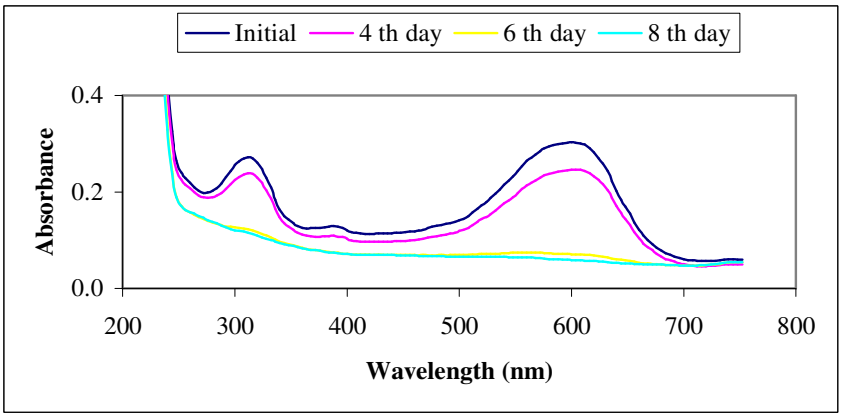


(c)

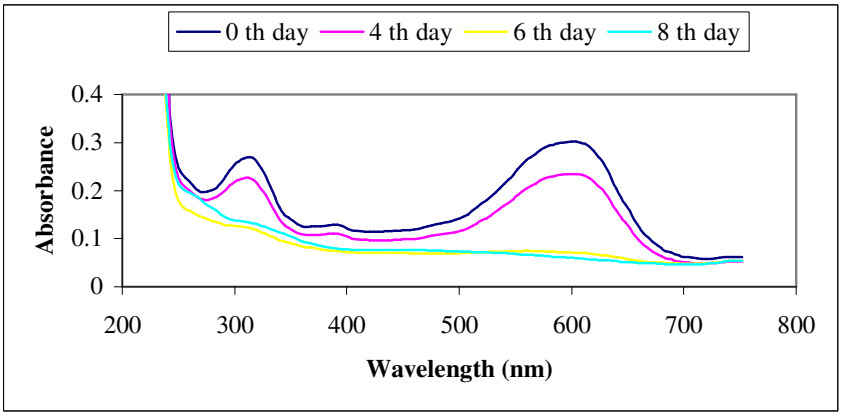
Figure 4.15. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 30% dilution (v/v) of a) RB-5, b) RB-5 and 0.1 g L^{-1} glucose and c) RB-5 and 0.2 g L^{-1} glucose.



(a)

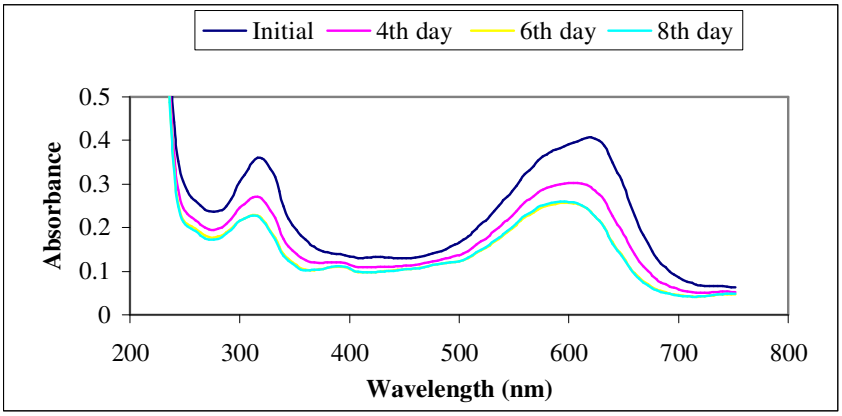


(b)

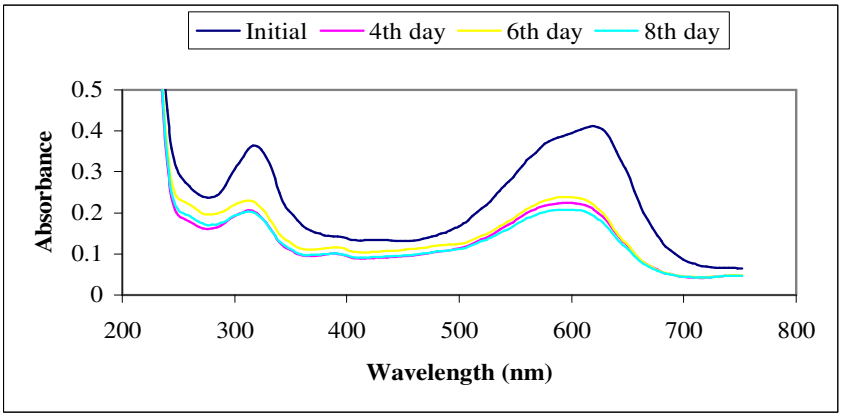


(c)

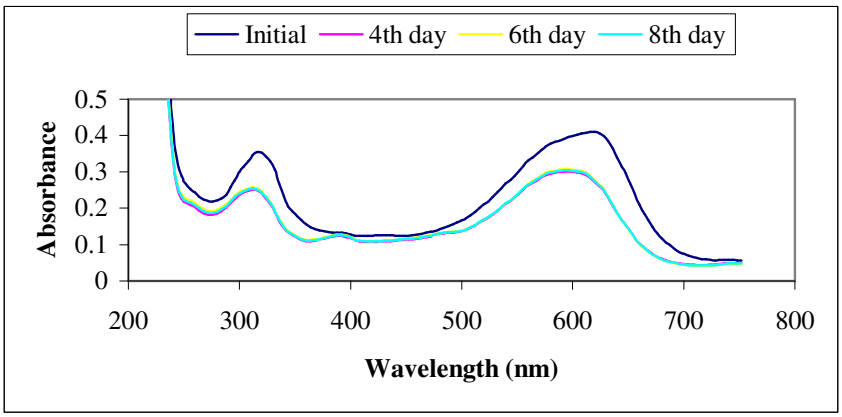
Figure 4.16. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 60% dilution (v/v) of a) RB-5, b) RB-5 and 0.1 g L^{-1} glucose and c) RB-5 and 0.2 g L^{-1} glucose.



(a)

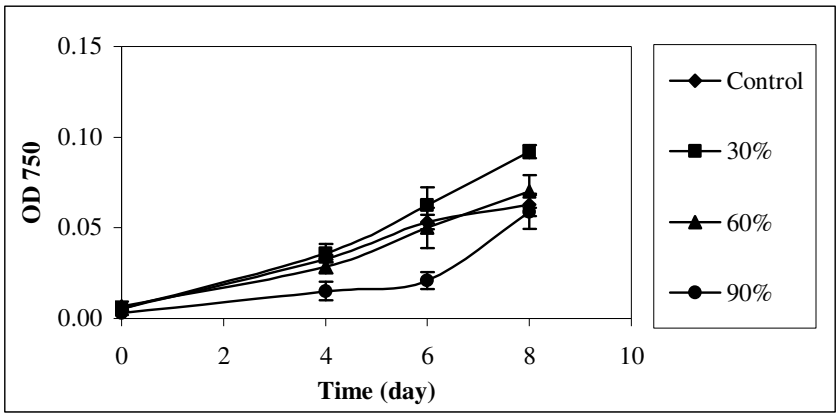


(b)

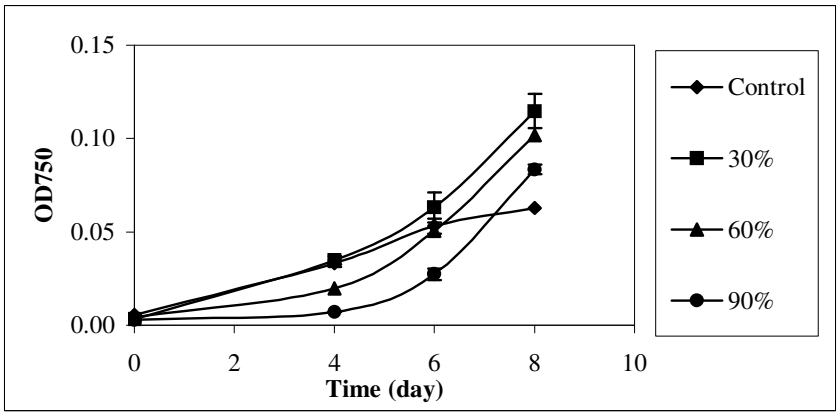


(c)

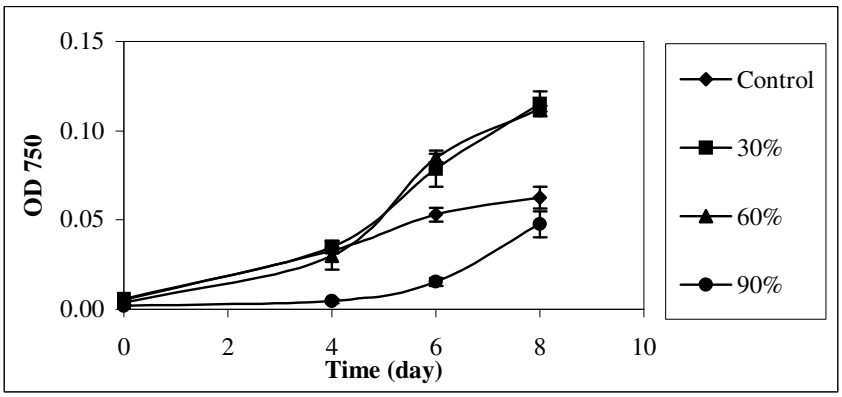
Figure 4.17. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 90% dilution (v/v) of a) RB-5, b) RB-5 and 0.1 g L^{-1} glucose and c) RB-5 and 0.2 g L^{-1} glucose.



(a)



(b)



(c)

Figure 4.19. Growth curves of *D. tertiolecta* exposed to a) RB-5 B) RB-5 and 0.1 g L^{-1} glycerol and c) RB-5 and 0.2 g L^{-1} glycerol.

Table 4.4. Variation in optical density at 750 nm and the 8-d IC_{25} and SC_{20} of *Dunaliella tertiolecta* exposed to RB-5 in the presence and absence of glycerol.

Control	% dilution (v/v)				IC_{25} % dilution (v/v)	TU*	SC_{20} % dilution (v/v)	TU**
	0	30	60	90				
REACTIVE BLACK 5								
AUC	0.21	0.40	0.18	0.04	62.58#±6.76##	1.59	8.66±1.15 51.33±1.33	11.53 1.94
CV	7.59	15.61	8.36	31.09				
Ig (%)		-92.45	12.26	79.40				
REACTIVE BLACK 5 with 0.1 g L^{-1} GLYCEROL								
AUC	0.21	0.32	0.23	0.14	83.50±2.82	1.19	4.66±0.57 57.66±2.51	21.42 1.73
CV	7.59	4.20	3.76	5.53				
Ig (%)		-53.14	-11.32	33.49				
REACTIVE BLACK 5 with 0.2 g L^{-1} GLYCEROL								
AUC	0.21	0.34	0.35	0.08	84.09±4.06	1.18	5.00±1.00 73.33±1.52	20.00 1.36
CV	7.59	2.42	4.70	8.47				
Ig (%)		-61.95	-64.93	62.10				

AUC = Mean values from repeated tests (n=3),
CV = Coefficient of variation
Ig (%) = % growth inhibition/stimulation; negative values show stimulation of growth with respect to the control
IC_{25} = 25% Inhibition concentration
SC_{20} = 20% Stimulation concentration
TU = Toxic Unit in terms of IC_{25}
# = 25% Inhibition concentration calculated from ICp method and the rest was calculated from the curve fitting analysis
= ± values show standard deviation
* = Toxic Unit in terms of IC_{25}
** = Toxic Unit in terms of SC_{20}

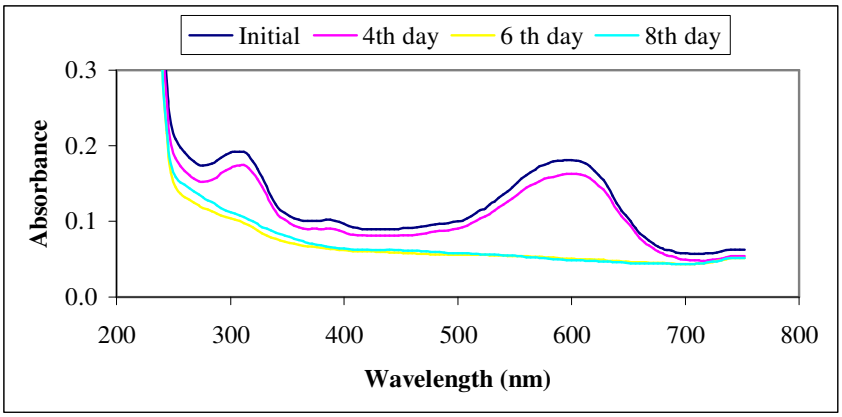
RB-5 were considered, addition of 0.1 g L^{-1} and 0.2 g L^{-1} glycerol as a carbon and energy source increased the stimulatory effect of RB-5 significantly (p<0.05) compared to the single exposure of RB-5 and the control.

TU values of RB-5, RB-5with 0.1 g L^{-1} glycerol and RB-5 with 0.2 g L^{-1} glycerol were 1.59, 1.19 and 1.18, respectively, when TU values calculated on the basis of 8-d IC_{25}. Consequently, RB-5, RB-5 with 0.1 g L^{-1} glycerol and RB-5 with 0.2 g L^{-1} glycerol were moderately toxic (TU>1.0, Villegas-Navarro et al., 2001). On the other hand, TU values of RB-5, RB-5 with 0.1 g L^{-1} glycerol and RB-5 with 0.2 g L^{-1} glycerol were in the range of 1.89-14.28, 1.71-15.79 and 1.76-13.05, respectively when TU values were evaluated on the basis of 8-d SC_{20}. This reflects that RB-5 with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol and without glycerol were moderately toxic (TU>1.0) at high dilutions but they were very toxic (TU≥3) at low dilutions. TU in terms of SC_{20} and IC_{25} indicating that RB-5 may cause adverse effects in an aquatic environment by stimulation of algal growth at low concentrations and an inhibition of algal growth at high concentrations. These adverse effects were discussed in section 4.2.1.

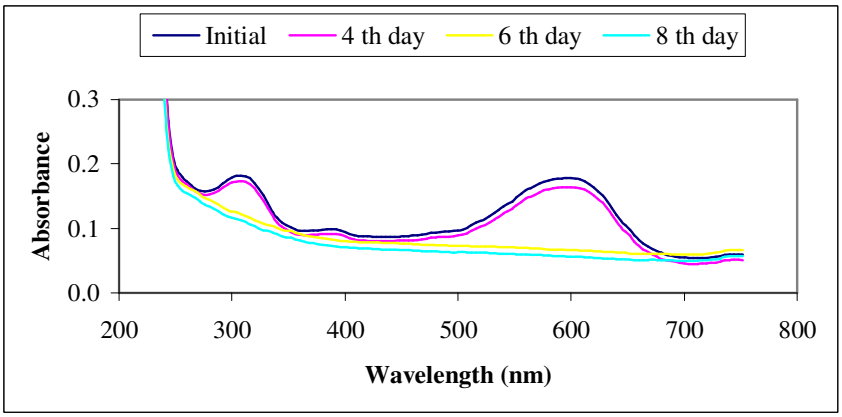
4.3.5. Decolorization of Reactive Black 5 with *Dunaliella tertiolecta* in the Presence and Absence of Glycerol

The decolorization of RB-5 with and without glycerol was tested for dilutions ranging from 30% (v/v) to 90% (v/v). The cultures were monitored by considering parameters like, change in the absorbance of the prepared dye solutions, decolorization period and the effect of glycerol. The UV-Vis spectra of all tested samples collected from the batch culture during a reaction period of eight days of incubation are shown in Figures from 4.20 to 4.22 to be able to determine absorbance removal at the wavelengths which RB-5 gives maximum absorbance.

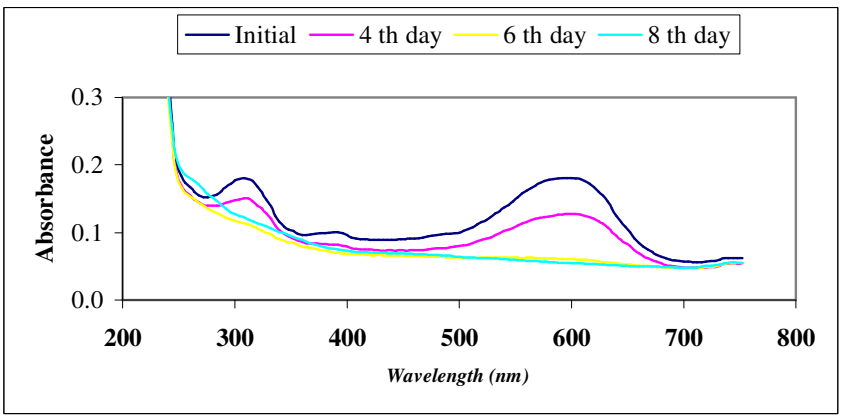
Concentration-dependent decolorization was observed similar to DB. The time overlaid UV-Vis spectra of test samples of RB-5 collected during the decolorization experiments are shown in Figures from 4.20 to 4.22. The intensity of the characteristic absorption peaks of RB-5 at 600 and 316 nm decreased drastically within six days for 30%



(a)

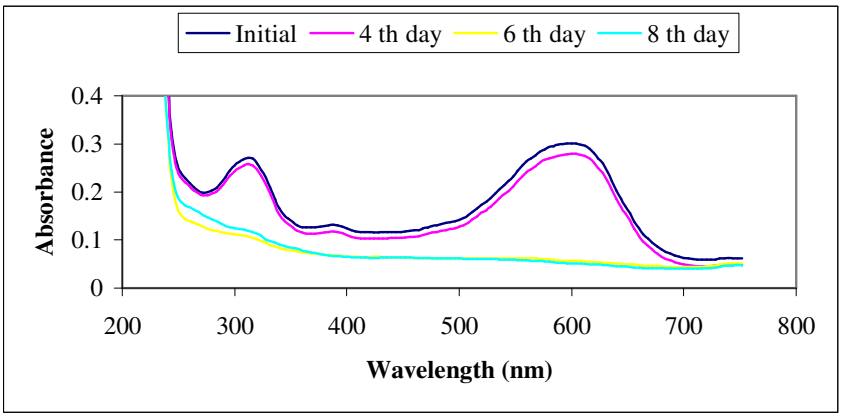


(b)

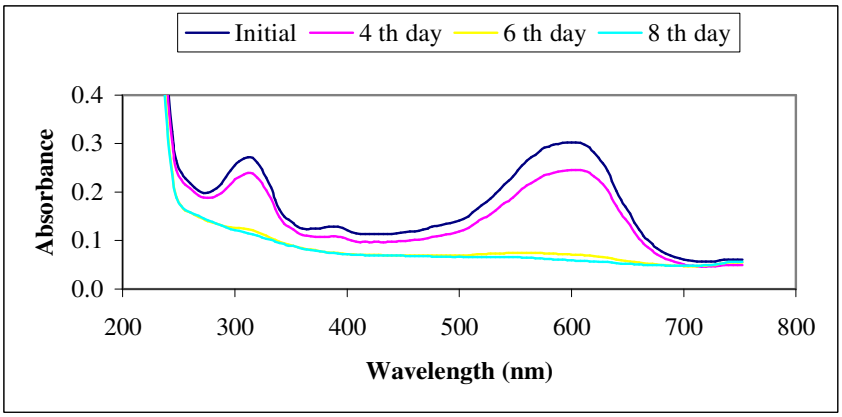


(c)

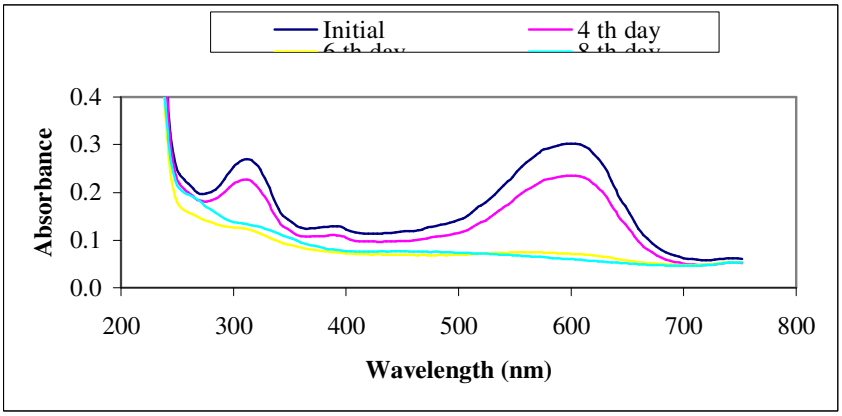
Figure 4.20. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 30% dilution (v/v) of a) RB-5, b) RB-5 and 0.1 g L^{-1} glycerol and c) RB-5 and 0.2 g L^{-1} glycerol.



(a)

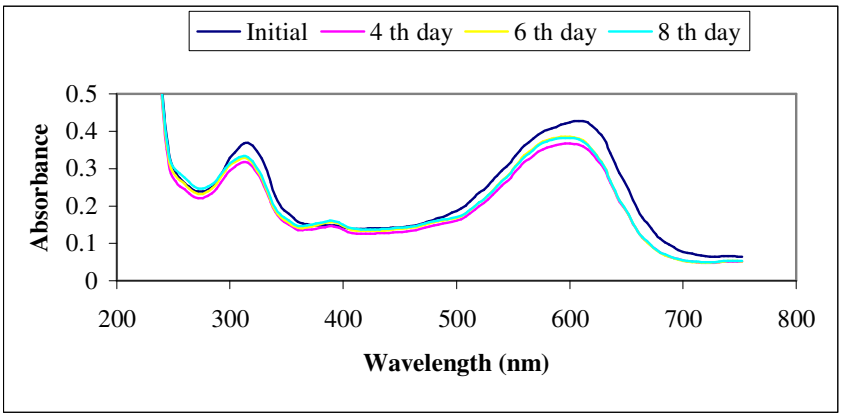


(b)

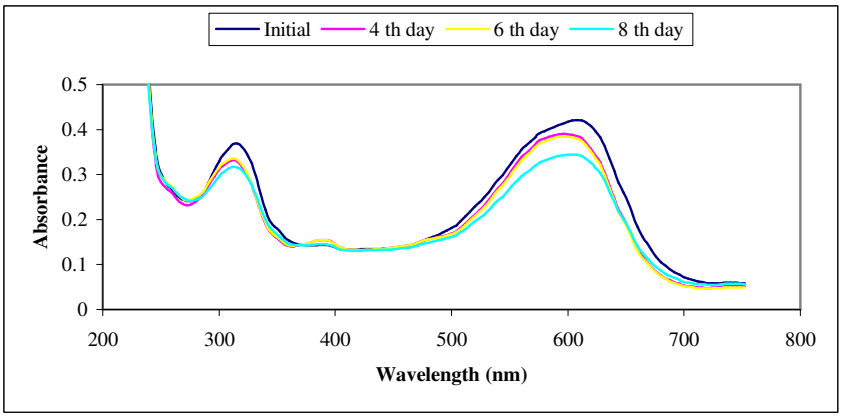


(c)

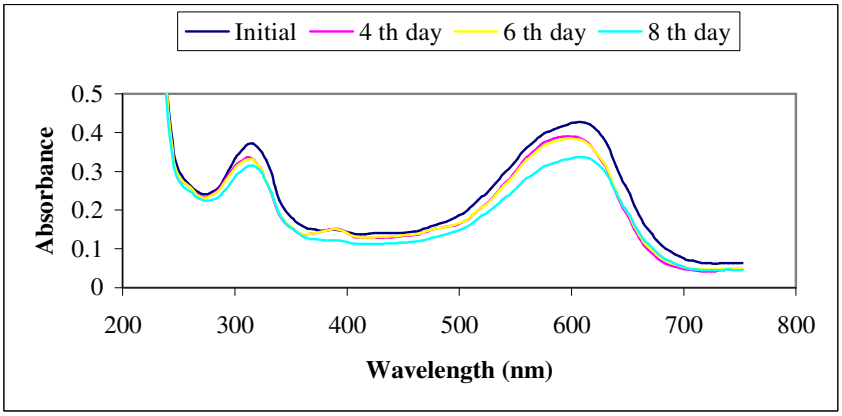
Figure 4.21. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 60% dilution (v/v) of a) RB-5, b) RB-5 and 0.1 g L^{-1} glycerol and c) RB-5 and 0.2 g L^{-1} glycerol.



(a)



(b)



(c)

Figure 4.22. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 90% dilution (v/v) of a) RB-5, b) RB-5 and 0.1 g L^{-1} glycerol and c) RB-5 and 0.2 g L^{-1} glycerol.

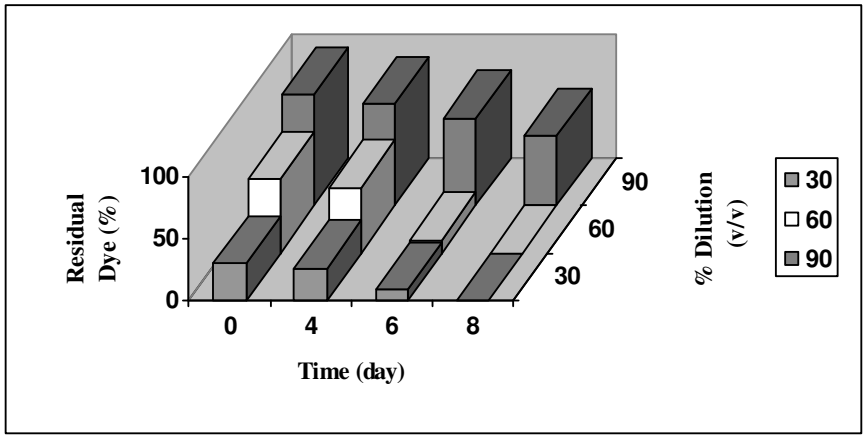
and 60% (v/v). *D. tertiolecta* decolorized the RB-5 at low dilutions (30% and 60% dilutions (v/v)) resulting in complete decolorization during six days of incubation although there was negligible decolorization within the first four days (Figures 4.20 and 4.21 (a) and (b)). While addition of 0.1 g L^{-1} glycerol did not change the decolorization time of RB-5 for 30% (v/v), approximately 35-45% decrease in absorbance at maximum wavelengths (600 and 316 nm) for 30% of RB-5 with 0.2 g L^{-1} glycerol was observed within four days (Figure 4.20 (b) and (c), respectively). Similarly, when the batch culture was supplemented with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol, the intensity of characteristic adsorption peaks of RB-5 at 600 and 316 nm decreased almost by one half within four days for 60% (v/v) (Figure 4.21 (b) an d (c), respectively).

Decolorization results of RB-5 obtained with *D tertiolecta* culture was relatively unsatisfactory for 90% (v/v) (Figures 4.22) as stated in section 4.3.2. Addition of both concentartions of glycerol did not change the decolorization of RB-5 for 90% (v/v).

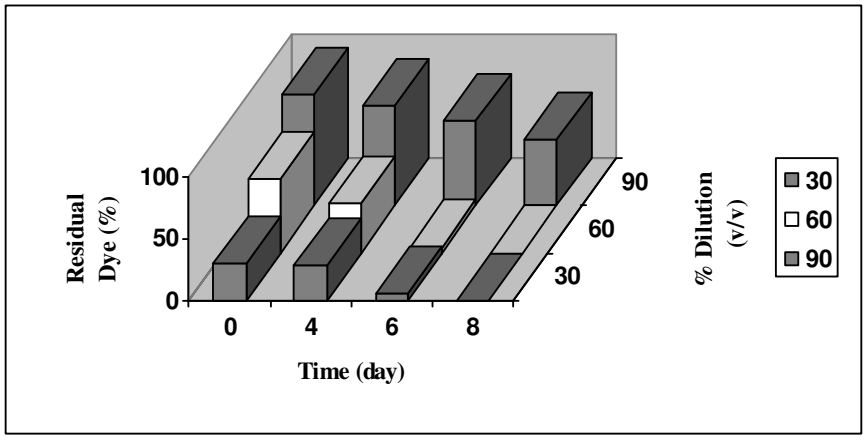
The observations indicated that low dilutions of RB-5 can be removed without a co-substrate by the algae. However, when batch culture was supplemented with 0.2 g L^{-1} glycerol, approximately a 35-45% decrease in absorbance at maximum wavelengths at 600 and 316 nm for 30% was observed within four days. Addition of 0.1g L^{-1} and 0.2 g L^{-1} glycerol also enhanced the decolorization of RB-5 for 60% (v/v) within four days. There was no significant difference between decolorization of RB-5 with and without glycerol for 90% (v/v). The amount of co-substrate used (0.1 g.L^{-1} and 0.2 g L^{-1} glycerol) may not be enough to enhance the decolorization of RB-5 for high dilution.

4.3.6. Color Changes during the Decolorization of Reactive Black 5 with and without Glucose

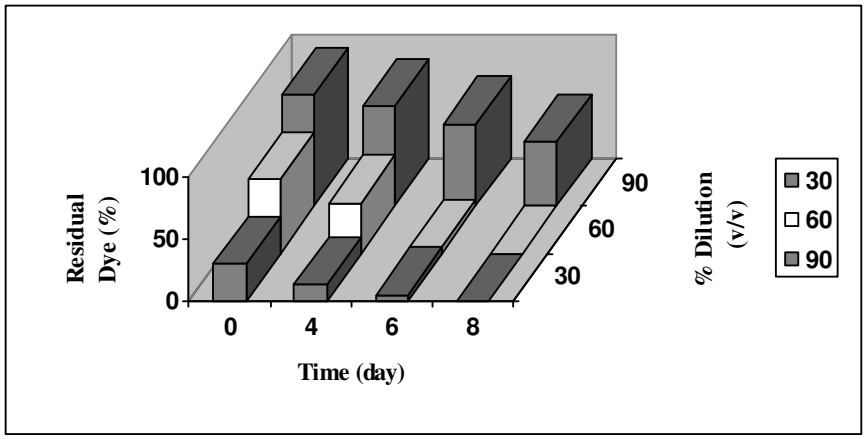
The color values of RB-5 with and without glycerol was also tested for dilutions ranging from 30% (v/v) to 90% (v/v) as a platinum cobalt (Pt-Co) unit in order to support the changes in the absorption spectra of RB-5 with *D. tertiolecta*. The variation of dye removal values are depicted in Figure 4.23 for RB-5 with and without glycerol for all studied dilutions. The results showed a positive correlation between color and spectral changes.


Residual Dye (%)
100
50
0
0
4
6
8
Time (day)
90
60
30
% Dilution (v/v)
30
60
90

(a)


Residual Dye (%)
100
50
0
0
4
6
8
Time (day)
90
60
30
% Dilution (v/v)
30
60
90

(b)


Residual Dye (%)
100
50
0
0
4
6
8
Time (day)
90
60
30
% Dilution (v/v)
30
60
90

(c)

Figure 4.23. Color changes in the medium containing RB-5 a) singly b) with 0.1 g L^{-1} glycerol c) with 0.2 g L^{-1} glycerol.

The increase in dye concentration resulted of the reduction in color removal efficiencies. Almost no color removal was observed with *D. tertiolecta* within the first four days at all studied dilutions. The color removal efficiencies were 13%, 12% and 9% for 30%, 60% and 90% (v/v) of RB-5 within four days, respectively. Then, color values decreased rather sharply within the next two days resulting in complete decolorization within six days of incubation for 30% and 60% (v/v). However, when batch culture was supplemented with 0.2 g L^{-1} glycerol, 55% color removal was accomplished for 30% (v/v) of RB-5 within four days. Additionally, when batch culture was supplemented with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol, color values decreased rather sharply within the first four days resulting in 33% and 31% color removal efficiencies for 60% (v/v), respectively. Decolorization result of RB-5 with and without glycerol obtained with *D. tertiolecta* culture were relatively unsatisfactory for 90% (v/v) during eight days of incubation which correspond to only 37%, 41% and 42% color removal efficiencies for RB-5, RB-5 with 0.1 g L^{-1} glycerol and RB-5 with 0.2 g L^{-1} glycerol, respectively.

4.3.7. pH Changes during Decolorization of Reactive Black 5 with and without Glucose and Glycerol

Changes in the pH value of the culture media during the growth period of algae exposed to RB-5 with and without glucose and glycerol are shown in Figures 4.24 and 4.25, respectively. Error bars show the mean standard deviation between triplicates. Range of pH differed notably between control and samples containing RB-5 with and without glucose and glycerol. The average pH change in blank samples was 0.33, 0.98, 1.51 and 1.80 unit for control and 30%, 60% and 90% (v/v), respectively. The pH of all the blanks decreased during eight days because of the dissolution of CO_2 and subsequent formation of H_2CO_3. Algal growth caused marked changes in the pH of the culture media, raising the low pH values (e.g., control and 30% (v/v), Figures 4.24 and 4.25 (a) and (b), respectively) and first lowering and then raising the high ones (e.g., 60 and 90% dilutions (v/v), Figures 4.24 and 4.25 (c) and (d), respectively. In fact, pH increases due to utilization of carbon dioxide and bicarbonate in photosynthesis and growth.

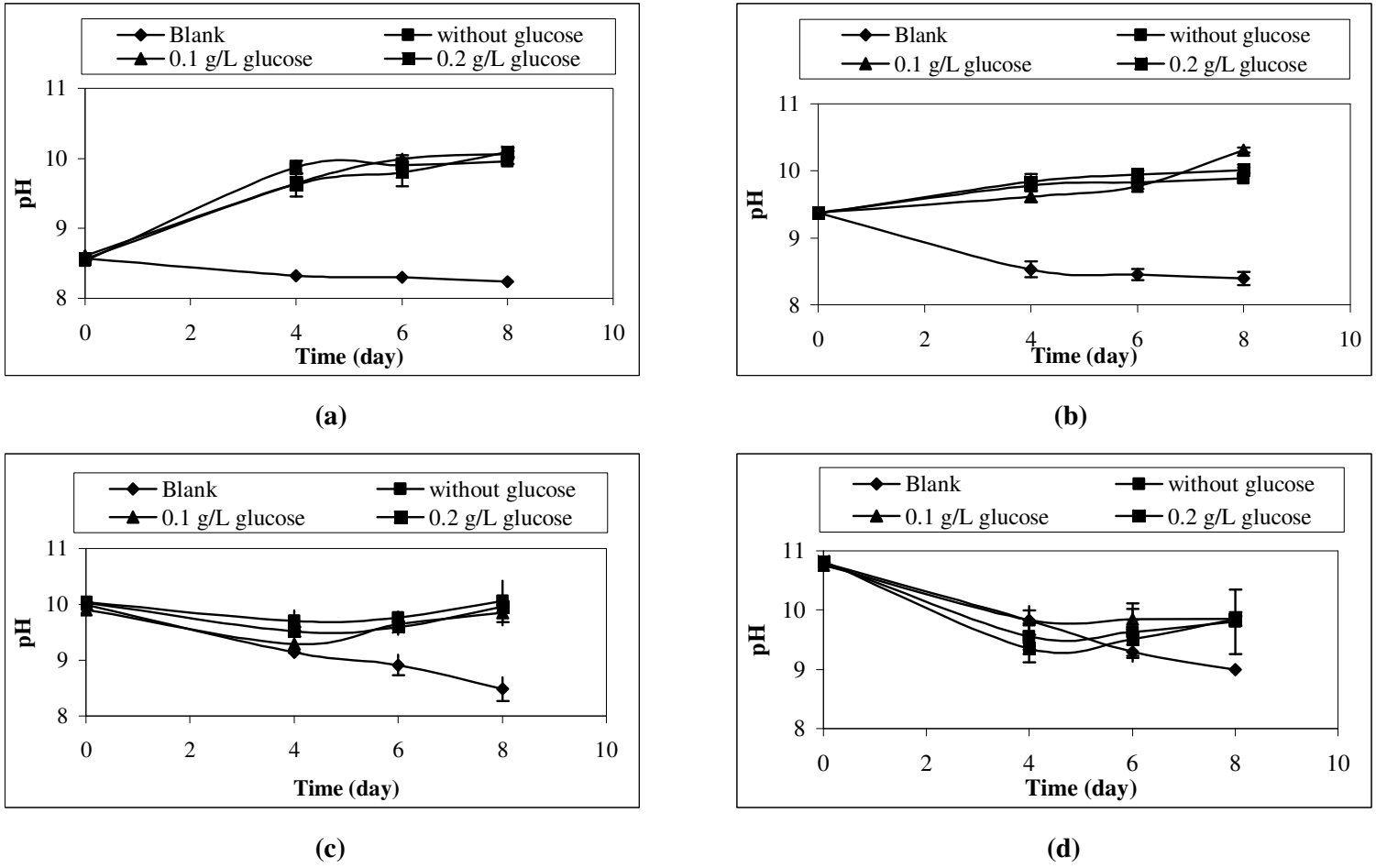


Figure 4.24. pH changes in the medium containing a) Control b) 30% RB-5, c) 60% RB-5 and d) 90% RB-5 with and without glucose.

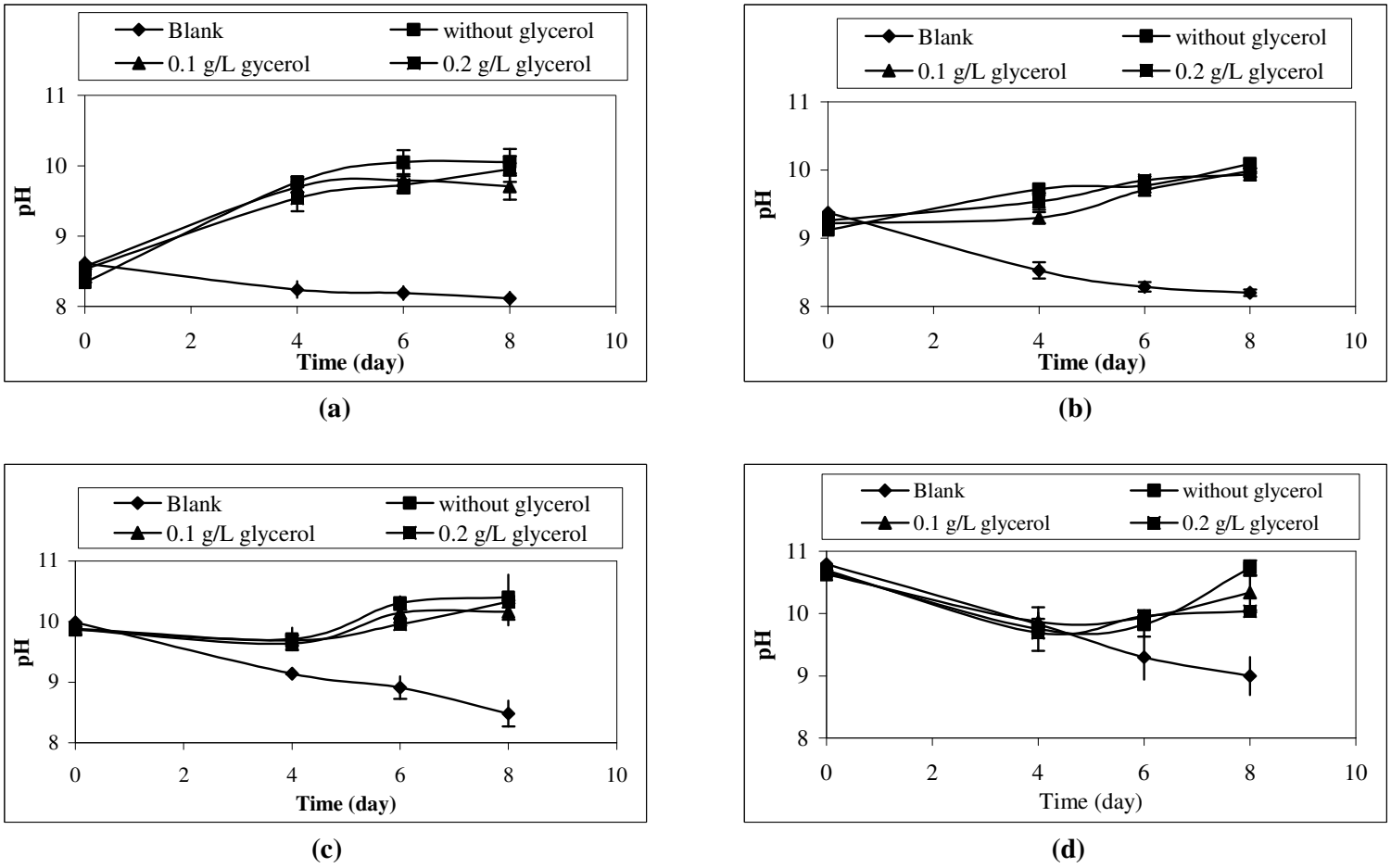


Figure 4.25. pH changes in the medium containing a) Control b) 30% RB-5, c) 60% RB-5 and d) 90% RB-5 with and without glycerol.

Within eight days all cultures exposed to RB-5 and glucose reached the pH range of 8.23-10.80. A sharp increase was observed in pH from 8.54 to 9.87 for the control and from 9.37 to 9.78 for RB-5 with and without glucose for 30% (v/v), then followed by almost a plateau. On the other hand, pH decreased in the test vessels containing 60% and 90% (v/v) during the first four days of growth, but it rose afterwards. While average 0.44 and 1.35 pH unit decreases were observed in the pH of the samples for 60 and 90% (v/v) dilutions with and without glucose, the average increase in pH for the same dilutions was 0.69 and 1.03 unit, respectively.

The same increasing and decreasing trend was also observed for the pH of the flasks containing RB-5 with and without glycerol for the control and all dilutions (30%, 60% and 90% (v/v)). Within eight days all cultures reached the pH range of 8.11-10.79. A sharp increase was observed in pH from 8.34 to 9.77 for control and from 9.12 to 9.72 for RB-5 with and without glycerol for 30% (v/v), then followed by almost a plateau. On the other hand, pH decreased in the test vessels containing 60% and 90% (v/v) during the first four days of growth, but it rose afterwards. While average 0.19 and 0.89 pH unit decreases were observed in the pH of the samples for 60% and 90% (v/v) with and without glycerol, the average increase in pH for the same dilutions was 0.62 and 0.70 units, respectively.

Glucose and glycerol did not change the initial pH and pH changing trend of the medium containing RB-5, significantly. The reasons for the increasing and decreasing values of pH were stated in Section 4.2.7.

4.4. Reactive Orange 69

4.4.1. Growth of *Dunaliella tertiolecta* Exposed to Reactive Orange 69 with and without Glucose

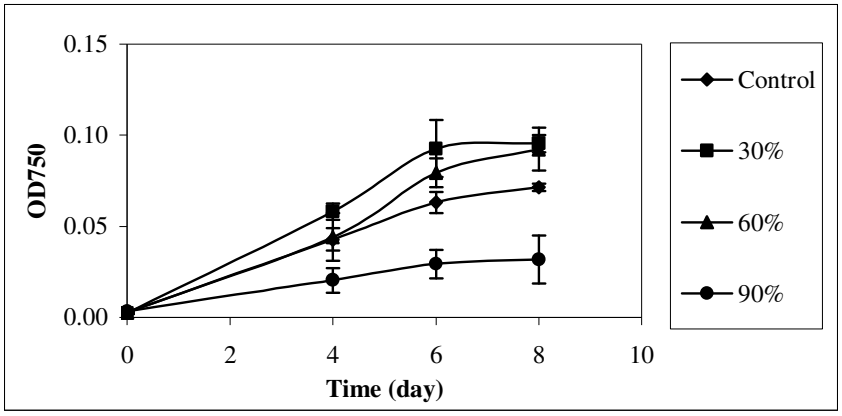
Reactive Orange 69 (RO-69) has monochlorodifluoropyrimidine as a reactive group and has the second highest concentration in the dye bath recipe. To evaluate the effect of toxicity of the RO-69 on the growth of *Dunaliella tertiolecta* batch studies were conducted with the same dilution of DB used in glucose experiments Growth curves of *D. tertiolecta* exposed to different dilution ratios of RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose and

without glucose were given in Figure 4.26. Error bars show the mean standard deviation between triplicates. All studied dilutions (% (v/v)) revealed a significant difference from the control ($p<0.05$).

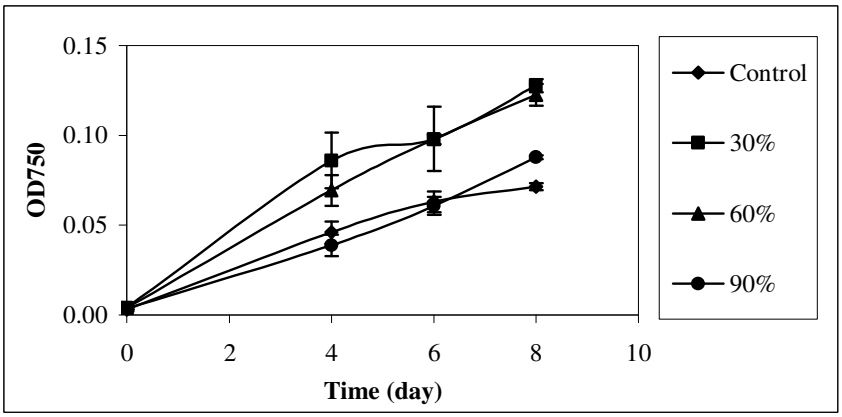
Algal growth was observed during the first eight days of incubation. The response of algae exposed to RO-69 was similar to that of RB-5. Algal growth was promoted at low dilutions of the RO-69 and the biomass increased with the increase in number of days. A lag phase duration (four days) was observed to be almost the same at all dilutions tested (30, 60, and 90% dilutions (v/v)). On the other hand, in the exponential phase, the growth of algae exposed to RO-69 with and without glucose at all dilutions except 90% (v/v) was higher than that of the control. However, addition of both 0.1 g L^{-1} and 0.2 g L^{-1} glucose resulted in stimulation on the growth of algae for all dilutions compared to the single exposure of RO-69 and the control (Figure 4.26 and Table 4.5). The growth of algae showed a decreasing trend with increasing dye concentration. Reduction in cell growth may result from the toxicity of dyes to algae through the inhibition of metabolic activities as stated in section 4.2.1

While hormetic response was observed in RO-69, only stimulation was observed for RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose for all dilutions (Table 4.6). In other words, glycerol eliminated the inhibitory effects of RO-69 on the growth response of algae for all dilutions.

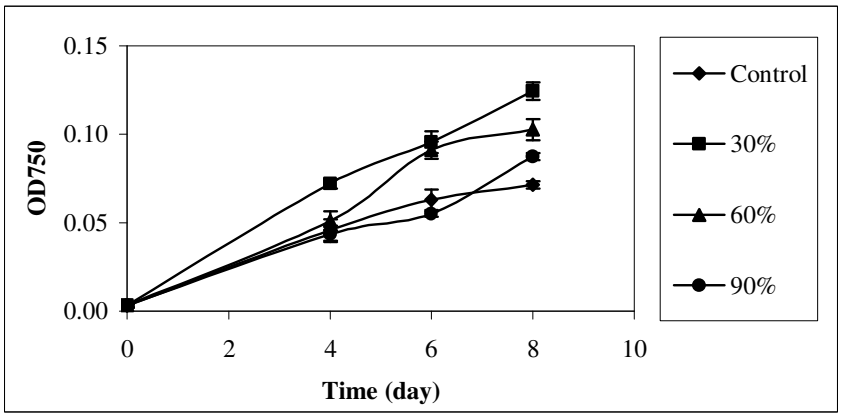
Calculated area under the growth curves (AUC) from optical density (OD_{750}) were tabulated in Table 4.5 together with the 8-d IC_{25} and SC_{20} and TU values. The 8-d SC_{20} values for RO-69, RO-69 with 0.1 g L^{-1} glucose and RO-69 with 0.2 g L^{-1} glucose were in the range of 7.75-67.57%, 5.40-83.13% and 3.34-84.45% (v/v), respectively. Inhibitory effect was observed at a higher dilution percentage. However, inhibitory effect was observed at a higher dilution percentage when algae exposed to only RO-69. RO-69 was toxic to algae with the 8-d IC_{25} value of 73.68±3.53% (v/v). Since both concentrations of glucose eliminated the inhibitory effect of RO-69 for the studied dilutions, the 8-d IC_{25} values couldn't be calculated for RO-69 in the presence of glucose. In other words, no toxicity of RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose was detectable with an IC_{25} of >100%. When the 8-d IC_{25} and SC_{20} values of RO-69 were considered, addition of 0.1 g L^{-}



(a)



(b)



(c)

Figure 4.26. Growth curves of *D. tertiolecta* exposed to a) RO-69 b) RO-69 and 0.1 g L^{-1} glucose and c) RO-69 and 0.2 g L^{-1} glucose.

Table 4.5. Variation in optical density at 750 nm and the 8-d IC_{25} and SC_{20} of *Dunaliella tertiolecta* exposed to RO-69 in the presence and absence of glucose.

Control	% dilution (v/v)				IC_{25} % dilution (v/v)	TU*	SC_{20} % dilution (v/v)	TU**
	0	30	60	90				
REACTIVE ORANGE 69								
AUC	0.30	0.49	0.36	0.13				
CV	2.07	0.23	4.23	23.54	73.68#±3.53##	1.35	7.75±1.78 67.57±7.00	12.92 1.47
Ig (%)		-61.92	-20.13	57.01				
REACTIVE ORANGE 69 with 0.1 g L^{-1} GLUCOSE								
AUC	0.30	0.55	0.50	0.32				
CV	2.07	1.50	6.82	15.06	>100	<1	5.40±1.83 83.13±0.19	18.54 1.20
Ig (%)		-76.75	-59.32	2.06				
REACTIVE ORANGE 69 with 0.2 g L^{-1} GLUCOSE								
AUC	0.30	0.54	0.41	0.33				
CV	2.07	1.40	3.86	1.67	>100	<1	3.34±0.49 84.45±1.06	29.89 1.18
Ig (%)		-79.26	-36.71	-0.92				

AUC = Mean values from repeated tests (n=3),
CV = Coefficient of variation
Ig (%) = % growth inhibition/stimulation; negative values show stimulation of growth with respect to the control
IC_{25} = 25% Inhibition concentration
SC_{20} = 20% Stimulation concentration
TU = Toxic Unit in terms of IC_{25}
# = 25% Inhibition concentration calculated from ICp method and the rest was calculated from the curve fitting analysis
= ± values show standard deviation
* = Toxic Unit in terms of IC_{25}
** = Toxic Unit in terms of SC_{20}

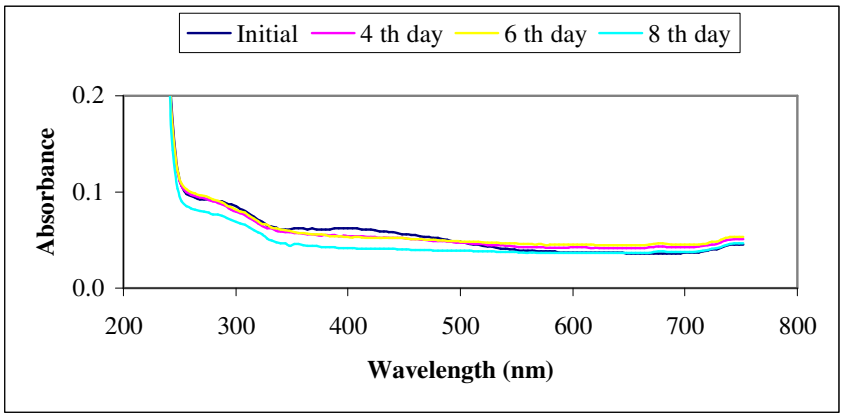
^{1} and 0.2 g L^{-1} glucose as carbon and energy source increased the stimulatory effect of RO-69 significantly (p<0.05) single exposure of RO-69 and the control.

TU value of RO-69 was 1.35, when TU values calculated on the basis of 8-d IC_{25}. Consequently, RO-69 was moderately toxic (TU>1.0, Villegas-Navarro et al., 2001). However, RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose was not toxic with TU<1.0 (Table 4.5). On the other hand, TU values of RO-69, RO-69 with 0.1 g L^{-1} glucose and 0.2 g L^{-1} glucose were in the range of 1.63-20.35, 1.20-18.54 and 1.18-29.89, respectively when TU values were evaluated on the basis of 8-d SC_{20}. This reflects that RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose and without glucose were moderately toxic (TU>1.0) at high dilutions but they were very toxic (TU≥3) at low dilutions. TU in terms of SC_{20} and IC_{25} indicating that RO-69 may cause adverse effects in an aquatic environment by stimulation of algal growth at low concentrations and an inhibition of algal growth at high concentrations as stated in Section 4.2.1. RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose cause only stimulatory effect on the growth of algae.

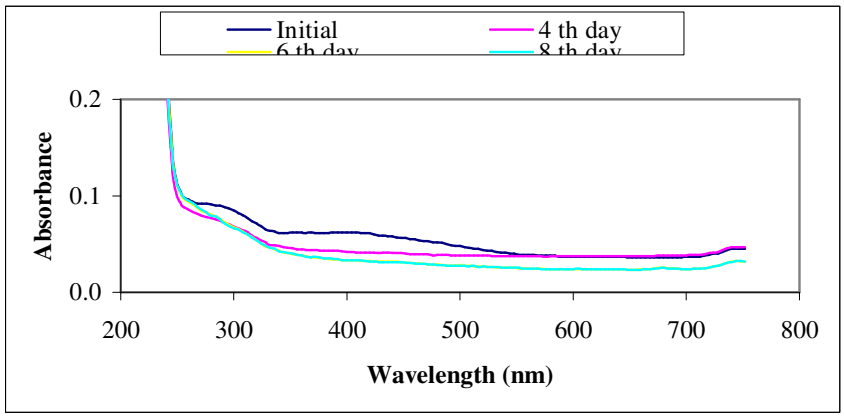
4.4.2. Decolorization of Reactive Orange 69 with *Dunaliella tertiolecta* in the Presence and Absence of Glucose

The decolorization of RO-69 with and without glucose was tested for dilutions ranging from 30% (v/v) to 90% (v/v). The cultures were monitored by considering parameters like, change in the absorbance of the prepared dye solutions, decolorization period and the effect of glucose. The UV-Vis spectra of all tested samples collected from the batch culture during a reaction period of eight days of incubation are shown in Figures from 4.27 to 4.29 to be able to determine absorbance removal at the wavelengths which RO-69 gives maximum absorbance.

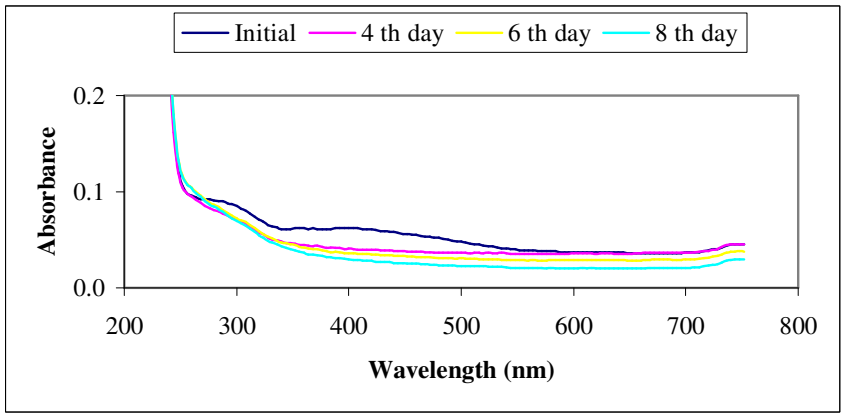
Concentration-dependent decolorization was observed similar to DB and RB-5. The time overlaid UV-Vis spectra of test samples of RO-69 collected during the decolorization experiments are shown in Figures from 4.27 to 4.29. The intensity of the characteristic absorption peaks of RO-69 at 424 and 296 nm decreased drastically within four days for 30% and 60% (v/v) dilutions. *D. tertiolecta* decolorized the RO-69 at low dilutions (30% and 60% dilutions (v/v)) resulting in complete decolorization during six days of incubation



(a)

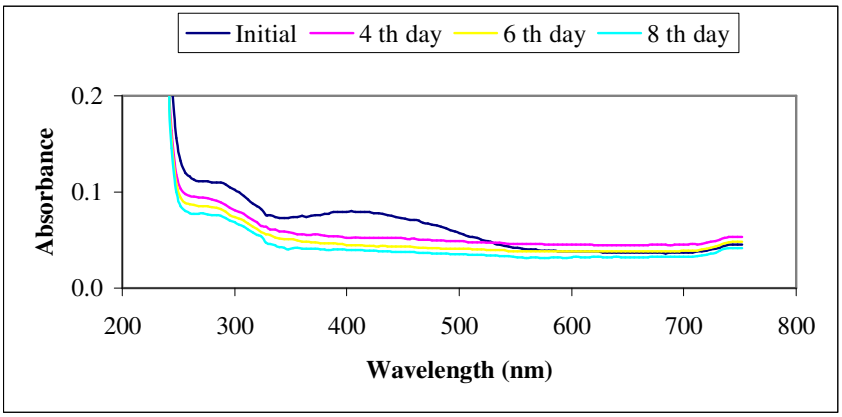


(b)

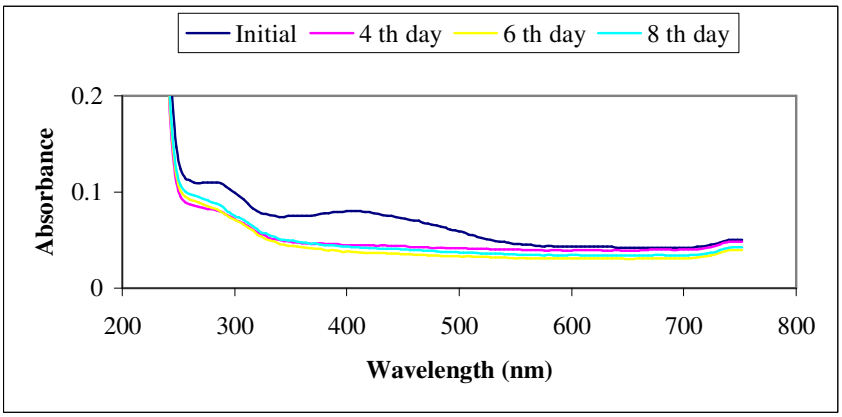


(c)

Figure 4.27. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 30% dilution (v/v) of a) RO-69, b) RO-69 and 0.1 g L^{-1} glucose and c) RO-69 and 0.2 g L^{-1} glucose.



(a)



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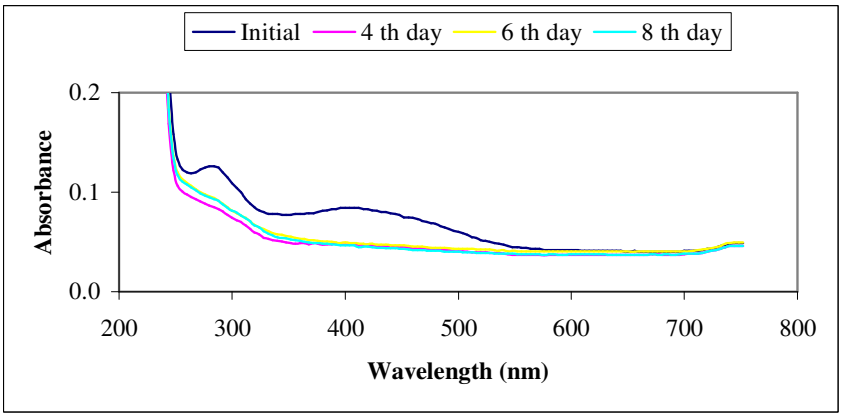
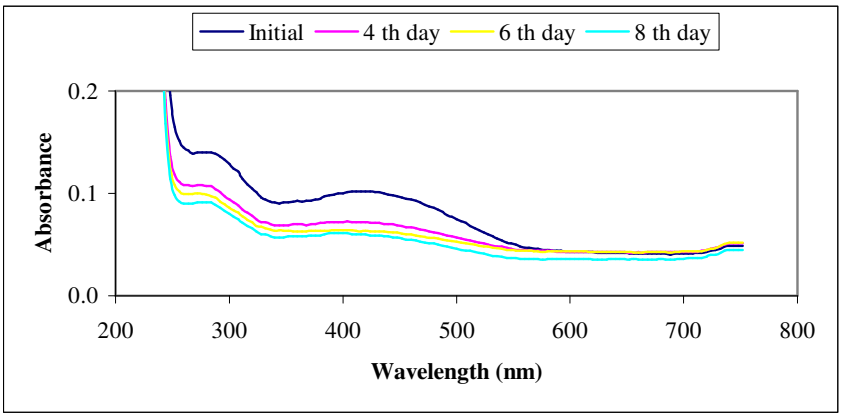
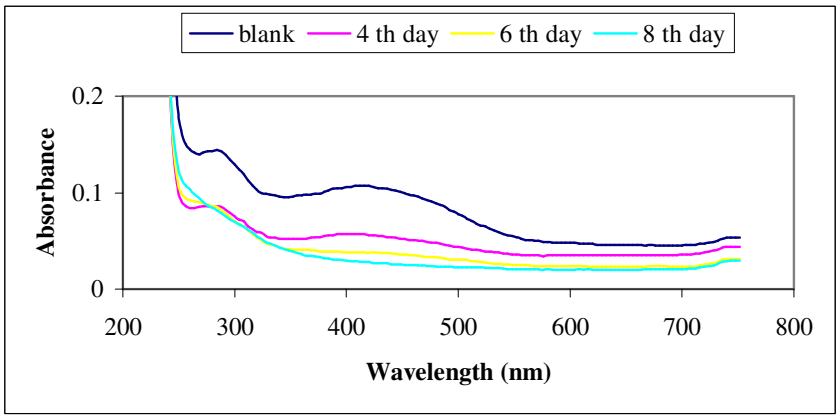


(c)

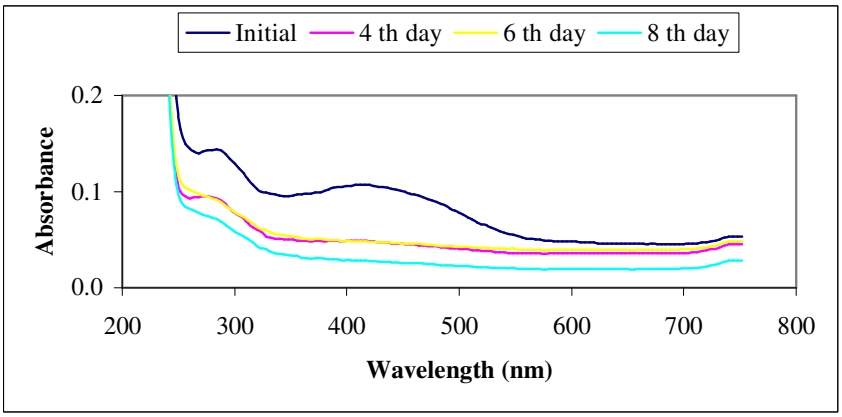
Figure 4.28. UV-vis spectral scans from the batch cultures of *D. tertiolecta* containing 60% dilution (v/v) of a) RO-69, b) RO-69 and 0.1 g L^{-1} glucose and c) RO-69 and 0.2 g L^{-1} glucose.



(a)



(b)



(c)

Figure 4.29. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 90% dilution (v/v) of a) RO-69, b) RO-69 and 0.1 g L^{-1} glucose and c) RO-69 and 0.2 g L^{-1} glucose.

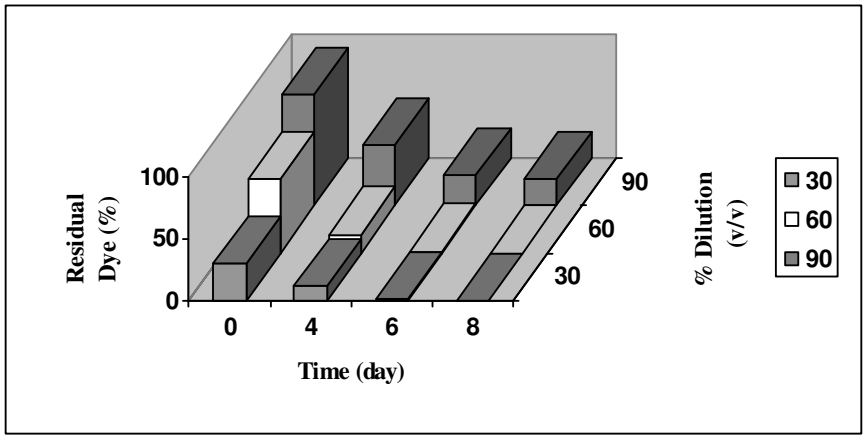
(Figures 4.27 and 4.28 (a)). Addition of 0.1 and 0.2 g L^{-1} glucose decreased the decolorization time of RO-69 for 30% and 60% (v/v) with *D. tertiolecta* from six days to four days (Figures 4.27 and 4.28 (b) and (c)).

Approximately a 45% decrease in absorbance at maximum wavelengths (424 and 296 nm) for 90% (v/v) with and without glucose was observed within four days, complete decolorization was not obtained with this culture exposed to only RO-69 even by extending the exposure period to eight days. However, when the batch culture was supplemented with 0.1 g L^{-1} and 0.2 g L^{-1} glucose, complete decolorization of RO-69 for 90% (v/v) was accomplished within six days (Figure 4.29 (b) and (c), respectively). Addition of 0.2 g L^{-1} glucose to 90% (v/v) lead to a drastic decrease at 424 nm compared to 0.1 g L^{-1} glucose within four days.

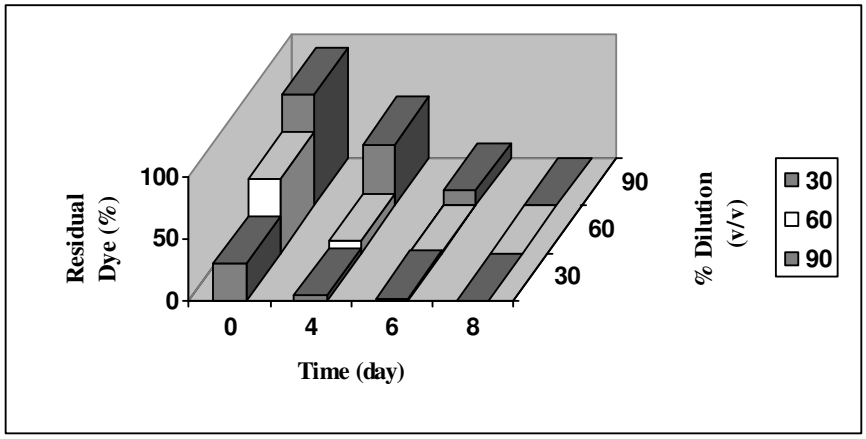
The observations indicated that low dilutions of RO-69 can be removed completely without a co-substrate by the algae. The color removal efficiency was 77% for high concentration (90% (v/v)). However, when the batch culture was supplemented with 0.1 g L^{-1} and 0.2 g L^{-1} glucose, complete decolorization was accomplished within four days for 30% and 60% (v/v) and within six days for 90% (v/v). This result showed that *D. tertiolecta* could use RO-69 as a carbon source but an external carbon source addition is necessary to speed up the decolorization process.

4.4.3. Color Changes during the Decolorization of Reactive Orange 69 with and without Glucose

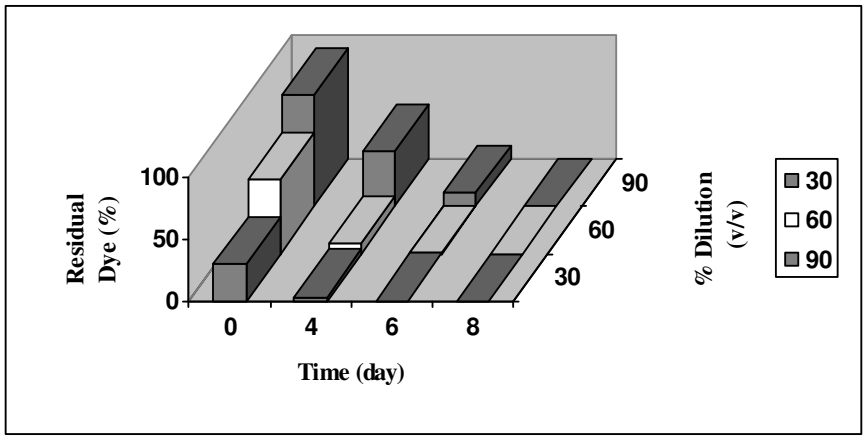
The color values of RO-69 with and without glucose was also tested for dilutions ranging from 30% (v/v) to 90% (v/v) as a platinum cobalt (Pt-Co) unit in order to support the changes in the absorption spectra of RO-69 with *D. tertiolecta*. The variation of dye removal values are depicted in Figure 4.30 for RO-69 with and without glucose for all studied dilutions. The results showed a positive correlation between color and spectral changes.



(a)



(b)



(c)

Figure 4.30. Color changes in the medium containing RO-69 a) singly b) with 0.1 g L^{-1} glucose c) with 0.2 g L^{-1} glucose.

The increase in dye concentration resulted of the reduction in color removal efficiencies. Color values decreased sharply within the first four days resulting in complete

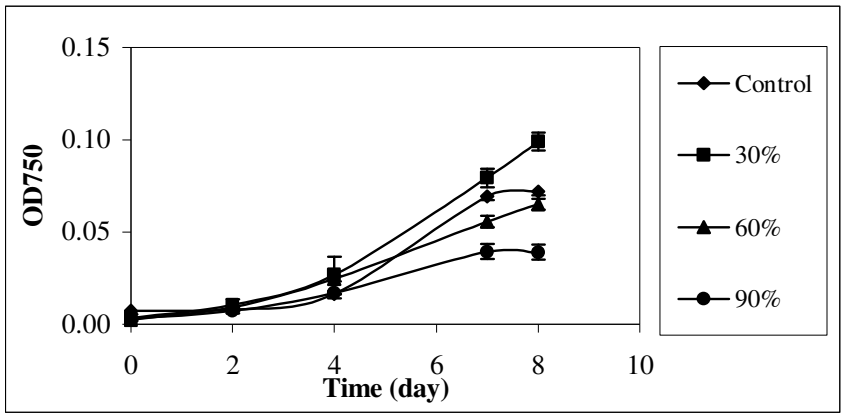
decolorization after eight days of incubation for 30% and 60% (v/v). The color removal efficiencies within four days were 62%, 87% and 88% for 30% (v/v) and 59%, 82% and 83% for 60% (v/v) of RO-69, RO-69 with 0.1 g L^{-1} glucose and RO-69 with 0.2 g L^{-1} glucose, respectively. Color removal efficiency of *D. tertiolecta* culture exposed to 90% (v/v) of RO-69 was 77% (Figure 4.30 (a)). However, complete color removal was accomplished for 90% (v/v) of RO-69 with the addition of glucose during eight days of incubation (Figure 4.30 (b) and (c)).

4.4.4. Growth of *Dunaliela tertiolecta* Exposed to Reactive Orange 69 with and without Glycerol

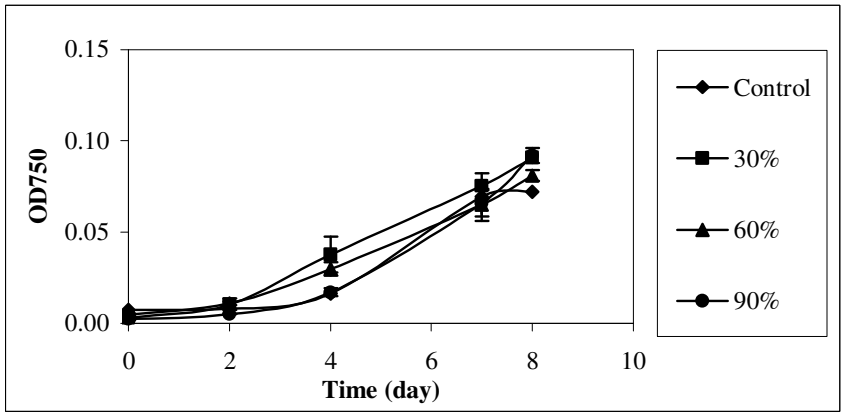
To evaluate the effect of toxicity of the RO-69 on the growth of *D. tertiolecta* batch studies were conducted with the same dilution of DB used in glucose experiments. Growth curves of *D. tertiolecta* exposed to different dilution ratios of RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol and without glycerol were given in Figure 4.31. Error bars show the mean standard deviation between triplicates. All studied dilutions (% (v/v)) revealed a significant difference from the control ($p<0.05$).

Algal growth was observed during the first eight days of incubation. The growth of algae showed a decreasing trend with increasing dye concentration. On the other hand, in the exponential phase, the growth of algae exposed to RO-69 was higher than that of the control at all dilutions except 90% (v/v) (Figure 4.31 (a)). However, addition of both 0.1 g L^{-1} and 0.2 g L^{-1} glycerol resulted in stimulation on the growth of algae for all dilutions compared to the single exposure of RO-69 and the control (Figure 4.31 and Table 4.6).

While hormetic response was observed in RO-69, only stimulation was observed for RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol for all dilutions (Table 4.6). In other words, glycerol eliminated the inhibitory effects of RO-69 on the growth response of algae for all dilutions.



(a)



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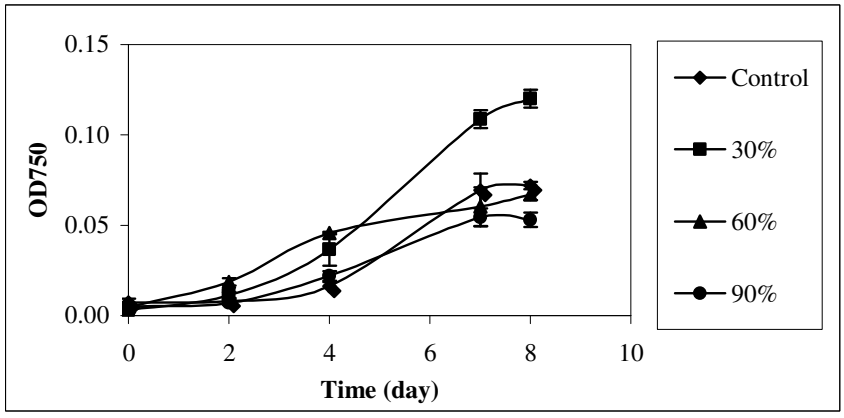


(c)

Figure 4.38. Growth curves of *D. tertiolecta* exposed to a) RO-69 B) RO-69 and 0.1 g L^{-1} glycerol and c) RO-69 and 0.2 g L^{-1} glycerol.

Table 4.6. Variation in optical density at 750 nm and the 8-d IC_{25} and SC_{20} of *Dunaliella tertiolecta* exposed to RO-69 in the presence and absence of glycerol.

Control	% dilution (v/v)				IC_{25} % dilution (v/v)	TU*	SC_{20} % dilution (v/v)	TU**
	0	30	60	90				
REACTIVE ORANGE 69								
AUC	0.11	0.18	0.14	0.08	73.68#±3.53##	1.35	7.75±1.78 67.57±7.00	12.92 1.47
CV	7.12	5.32	4.85	15.28				
Ig (%)		-59.48	-27.65	56.98				
REACTIVE ORANGE 69 with 0.1 g L^{-1} GLYCEROL								
AUC	0.11	0.21	0.16	0.13	>100	<1	3.54±0.21 83.65±4.17	28.24 1.19
CV	7.12	5.46	11.93	6.75				
Ig (%)		-79.60	-41.73	-17.09				
REACTIVE ORANGE 69 with 0.2 g L^{-1} GLYCEROL								
AUC	0.11	0.26	0.21	0.12	>100	<1	2.53±0.59 90.53±1.10	39.52 1.10
CV	7.12	7.41	15.33	7.65				
Ig (%)		-129.1	-83.16	-3.42				

AUC = Mean values from repeated tests (n=3),
CV = Coefficient of variation
Ig (%) = % growth inhibition/stimulation; negative values show stimulation of growth with respect to the control
IC_{25} = 25% Inhibition concentration
SC_{20} = 20% Stimulation concentration
TU = Toxic Unit in terms of IC_{25}
# = 25% Inhibition concentration calculated from ICp method and the rest was calculated from the curve fitting analysis
= ± values show standard deviation
* = Toxic Unit in terms of IC_{25}
** = Toxic Unit in terms of SC_{20}

Calculated area under the growth curves (AUC) from optical density (OD_{750}) were tabulated in Table 4.6 together with the 8-d IC_{25} and SC_{20} and TU values. The 8-d SC_{20} values observed for RO-69, RO-69 with 0.1 g L^{-1} glycerol and RO-69 with 0.2 g L^{-1} glycerol were in the range of 4.91-61.27%, 3.54-83.69% and 2.53-90.53% (v/v), respectively. However, inhibitory effect was observed at a higher dilution percentage when algae exposed to only RO-69. RO-69 was toxic to algae with the 8-d IC_{25} value of 73.68±3.53% (v/v). Since both concentrations of glycerol eliminated the inhibitory effect of RO-69 for the studied dilutions, the 8-d IC_{25} values couldn't be calculated for RO-69 in the presence of glycerol. In other words, no toxicity of RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol was detectable with an IC_{25} of >100%. When the 8-d IC_{25} and SC_{20} values of RO-69 were considered, addition of 0.1 g L^{-1} and 0.2 g L^{-1} glycerol as a carbon and energy source increased the stimulatory effect of RB-5 significantly compared to the single exposure of Ro-69 and the control (p<0.05) (Table 4.3).

TU value of RO-69 was 1.35 when TU values calculated on the basis of 8-d IC_{25}. Consequently, RO-69 was moderately toxic (TU>1.0, Villegas-Navarro et al., 2001). However, RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol was not toxic with TU<1.0 (Table 4.6). On the other hand, TU values of RO-69, RO-69 with 0.1 g L^{-1} glycerol and 0.2 g L^{-1} glycerol were in the range of 1.63-20.35, 1.19-28.24 and 1.10-39.52, respectively when TU values were evaluated on the basis of 8-d SC_{20}. This reflects that RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol and without glycerol were moderately toxic (TU>1.0) at high dilutions but they were very toxic (TU≥3) at low dilutions. TU in terms of SC_{20} and IC_{25} indicating that RO-69 may cause adverse effects in an aquatic environment by stimulation of algal growth at low concentrations and an inhibition of algal growth at high concentrations as stated in Section 4.2.1. RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol cause only stimulatory effect on the growth of algae.

4.4.5. Decolorization of Reactive Orange 69 with *Dunaliella tertiolecta* in the Presence and Absence of Glycerol

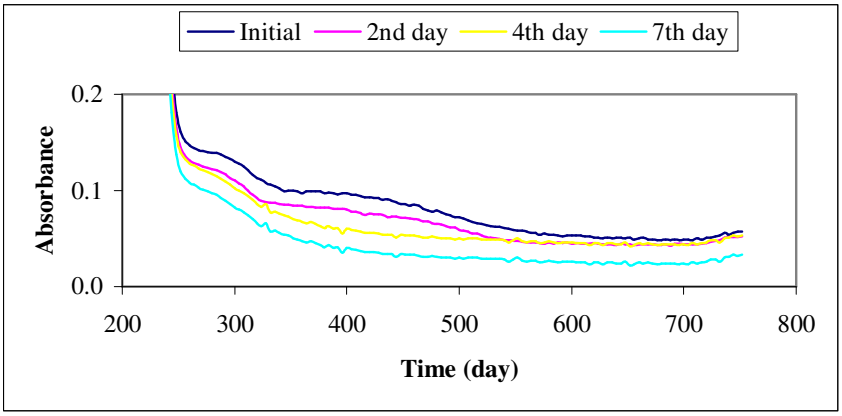
The decolorization of RO-69 with and without glycerol was tested for dilutions ranging from 30% (v/v) to 90% (v/v). The cultures were monitored by considering parameters like, change in the absorbance of the prepared dye solutions, decolorization

period and the effect of glycerol. The UV-Vis spectra of all tested samples collected from the batch culture during a reaction period of seven days of incubation are shown in Figures from 4.32 to 4.33 to be able to determine absorbance removal at the wavelengths which RO-69 gives maximum absorbance

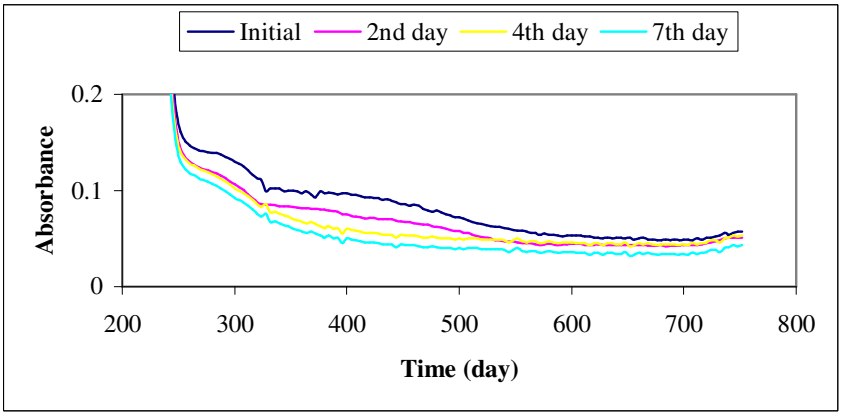
Concentration-dependent decolorization was observed similar to DB and RB-5. Since the concentration of RO-69 in DB recipe is lower than that of RB-5 and the addition of glycerol caused a complete decolorization for 30% and 60% (v/v) of RO-69 within four days, we measured intensity of characteristic absorption peaks starting from the second day. The time overlaid UV-Vis spectra of test samples of RO-69 collected during the decolorization experiments are shown in Figures from 4.32 to 4.33. The intensity of the characteristic absorption peaks of RO-69 at 424 and 296 nm decreased drastically within four days for 30% and 60% (v/v). *D. tertiolecta* decolorized RO-69 at low dilutions (30 and 60% dilutions (v/v)) resulting in complete decolorization during four days of incubation (Figures 4.32 and 4.33 (a)). Addition of 0.1 g L^{-1} and 0.2 g L^{-1} glycerol did not change the decolorization time of RO-69 for 30% and 60% (v/v) with *D. tertiolecta* (Figures 4.32 and 4.33 (a) and (b)).

Approximately a 37% decrease in absorbance at maximum wavelengths (424 and 296 nm) for 90% with and without glycerol was observed within four days, complete decolorization was not obtained with this culture exposed to only RO-69 even extending the exposure period to seven days. However, when batch culture exposed to 90% (v/v) of RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol, complete decolorization was accomplished within seven and four days, respectively,.

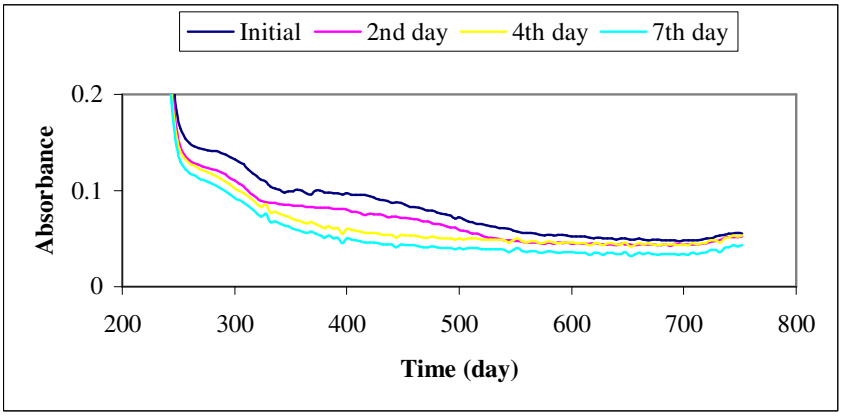
The observations indicated that low dilutions of RO-69 can be removed without co-substrate by the algae. In contrast, the color removal efficiency was 74% for high dilution (90% (v/v)). However, when the batch culture was supplemented with glycerol, complete decolorization of RO-69 for 90% (v/v) with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol was accomplished within seven and four days, respectively. This result showed that *D. tertiolecta* can use RO-69 as carbon source but external carbon source addition is necessary to speed up the decolorization.



(a)

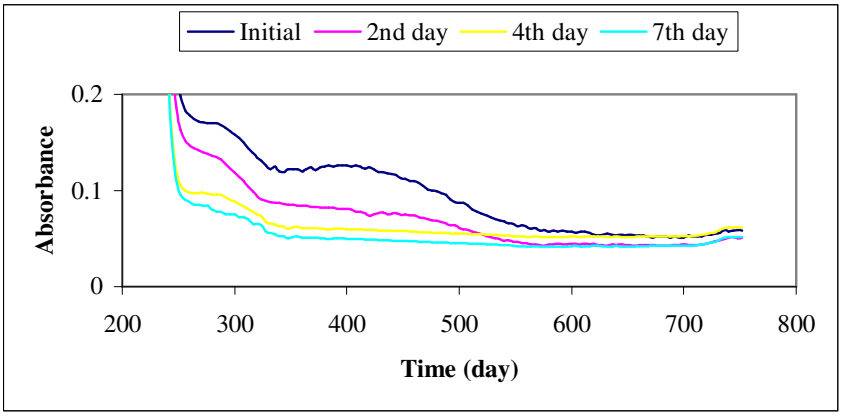


(b)

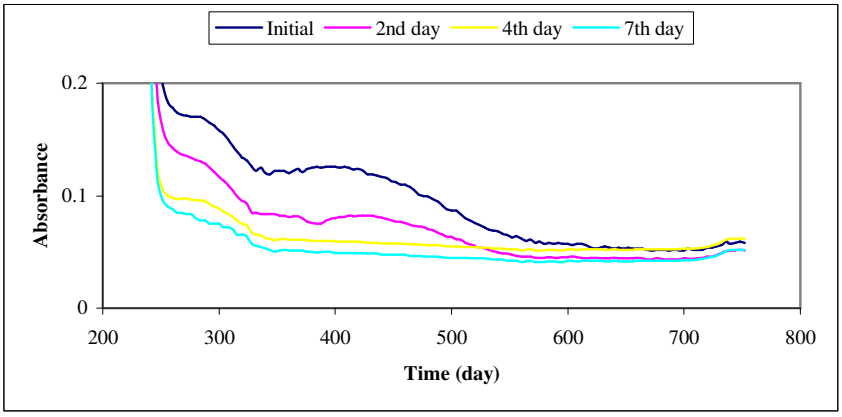


(c)

Figure 4.32. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 30% dilution (v/v) of a) RO-69, b) RO-69 and 0.1 g L^{-1} glycerol and c) RO-69 and 0.2 g L^{-1} glycerol.



(a)



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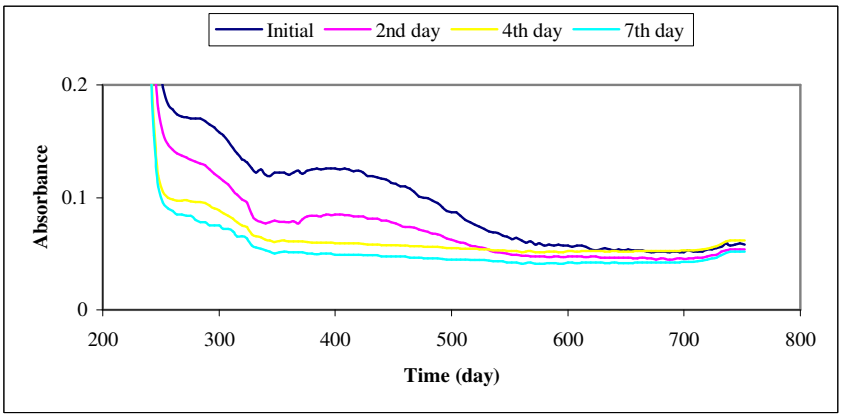
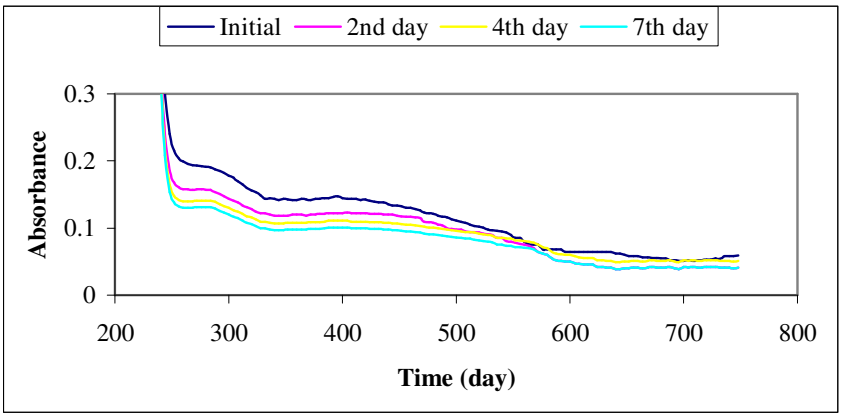
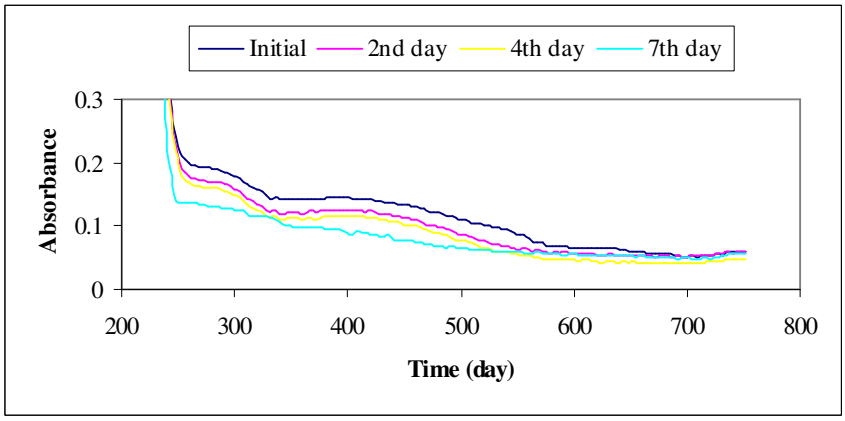


(c)

Figure 4.33. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 60% dilution (v/v) of a) RO-69, b) RO-69 and 0.1 g L^{-1} glycerol and c) RO-69 and 0.2 g L^{-1} glycerol.



(a)



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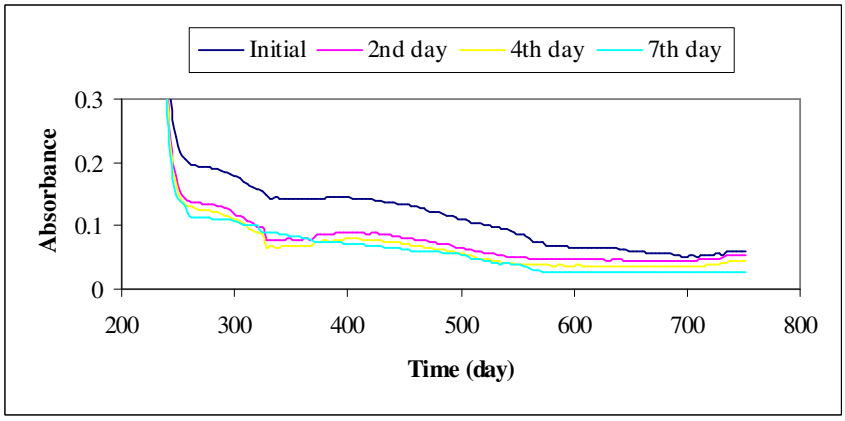


(c)

Figure 4.34. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 90% dilution (v/v) of a) RO-69, b) RO-69 and 0.1 g L^{-1} glycerol and c) RO-69 and 0.2 g L^{-1} glycerol.

4.4.6. Color Changes during Decolorization of Reactive Orange 69 with and without Glycerol

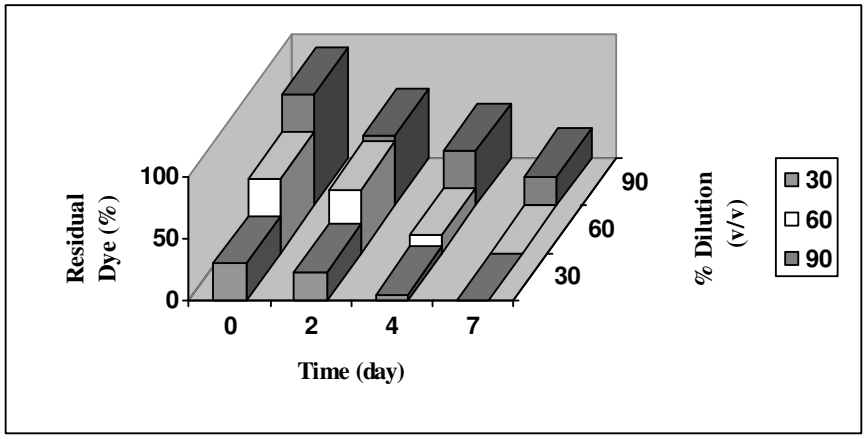
The color values of RO-69 with and without glycerol was also tested for dilutions ranging from 30% (v/v) to 90% (v/v) as a platinum cobalt (Pt-Co) unit in order to support the changes in the absorption spectra of RO-69 with *D. tertiolecta*. The variation of dye removal values are depicted in Figure 4.35 for RO-69 with and without glycerol for all studied dilutions. The results showed a positive correlation between color and spectral changes.

The increase in dye concentration resulted of the reduction in color removal efficiencies. Color values decreased sharply within the first four days resulting in complete decolorization during seven days of incubation for 30% and 60% (v/v). The color removal efficiencies within four days were 84%, 86% and 86% for 30% (v/v) and 75%, 80% and 79% for 60% (v/v) of RO-69, RO-69 with 0.1 g L^{-1} glycerol and RO-69 with 0.2 g L^{-1} glycerol, respectively (Figure 4.35). While the color removal efficiencies were 51%, 62% and 62% for 90% (v/v) of RO-69, RO-69 with 0.1 g L^{-1} glycerol and RO-69 with 0.2 g L^{-1} glycerol, respectively within four days, complete decolorization of 90% (v/v) of RO-69 was accomplished with the addition of glycerol during seven days of incubation (Figure 4.35). However, the color removal efficiency of single exposure of RO-69 was 74% during seven days of incubation (Figure 4.35).

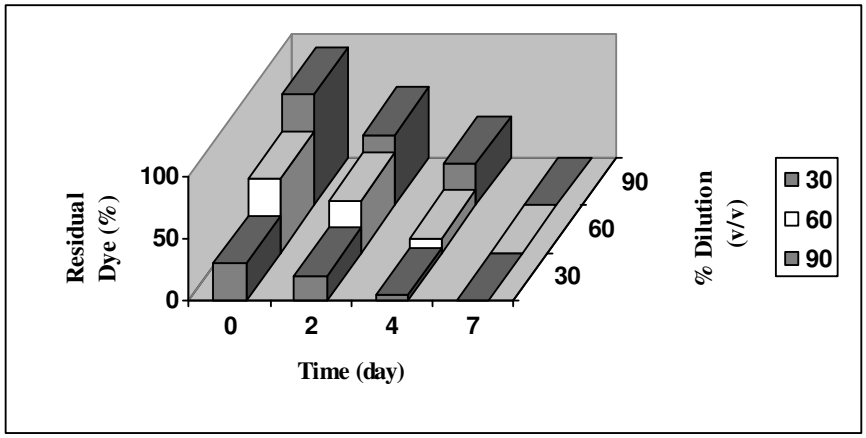
4.4.7. pH Changes During The Decolorization of Reactive Orange 69 with and without Glucose and Glycerol

Changes in the pH value of the culture media during the growth period of algae exposed to RO-69 with and without glucose and glycerol are shown in Figures 4.36 and 4.37, respectively. Error bars show the mean standard deviation between triplicates. Range of pH differed notably between control and samples containing RO-69 with and without glucose and glycerol. The average pH change in blank samples was 0.44, 0.98, 1.39 and 1.77 unit for control and 30%, 60% and 90% (v/v), respectively. The pH of all the blanks

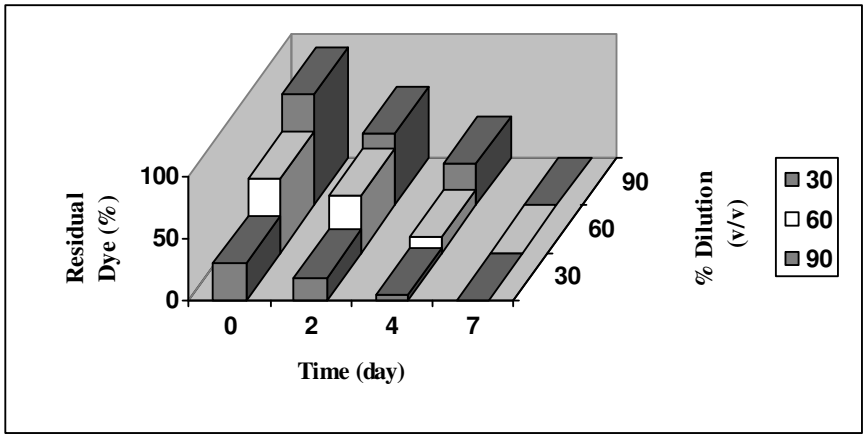
decreased during eight days because of the dissolution of CO_2 and subsequent formation of H_2CO_3. Algal growth caused marked changes in the pH of the culture media, raising the



(a)



(b)



(c)

Figure 4.35. Color changes in the medium containing RO-69 a) singly b) with 0.1 g L^{-1} glycerol c) with 0.2 g L^{-1} glycerol.

low pH values (e.g., control and 30% (v/v) dilution, Figures 4.36 and 4.37 (a) and (b), respectively) and first lowering and then raising the high ones (e.g., 60 and 90% (v/v) dilutions, Figures 4.36 and 4.37 (c) and (d), respectively). In fact, pH increases due to utilization of carbon dioxide and bicarbonate in photosynthesis and growth.

Within eight days all cultures exposed to RO-69 with and without glycerol reached the pH range of 8.22-11.14. A sharp increase was observed in pH from 8.63 to 9.95 for the control and from 9.30 to 9.85 for RO-69 with and without glucose for 30% (v/v) then, followed by almost a plateau. On the other hand, pH decreased in the test vessels containing 60% and 90% (v/v) during the first four days of growth, but it rose afterwards. While average 0.66 and 0.98 pH unit decreases were observed in the pH of the samples for 60% and 90% (v/v) with and without glucose, the average increase in pH for the same dilutions was 0.60 and 1.02 units, respectively.

The same increasing and decreasing trend was also observed for the pH of the flasks containing RO-69 with and without glycerol for the control and samples for 30%, 60% and 90% (v/v). Within seven days all cultures reached the pH range of 8.22-11.04. A sharp increase was observed in pH from 8.58 to 9.40 for control and from 9.60 to 10.01 for RO-69 with and without glycerol for 30% (v/v) then, followed by almost a plateau. On the other hand, pH decreased in the test vessels containing 60% and 90% (v/v) during the first four days of growth, but it rose afterwards. While average 0.65 and 1.02 pH unit decreases were observed in the pH of the samples for 60% and 90% (v/v) with and without glycerol, the average increase in pH for the same dilutions was 0.82 and 1.05 units, respectively.

Glucose and glycerol did not change the initial pH and pH changing trend of the medium containing RO-69, significantly. The reasons for the increasing and decreasing values of pH were stated in section 4.2.7.

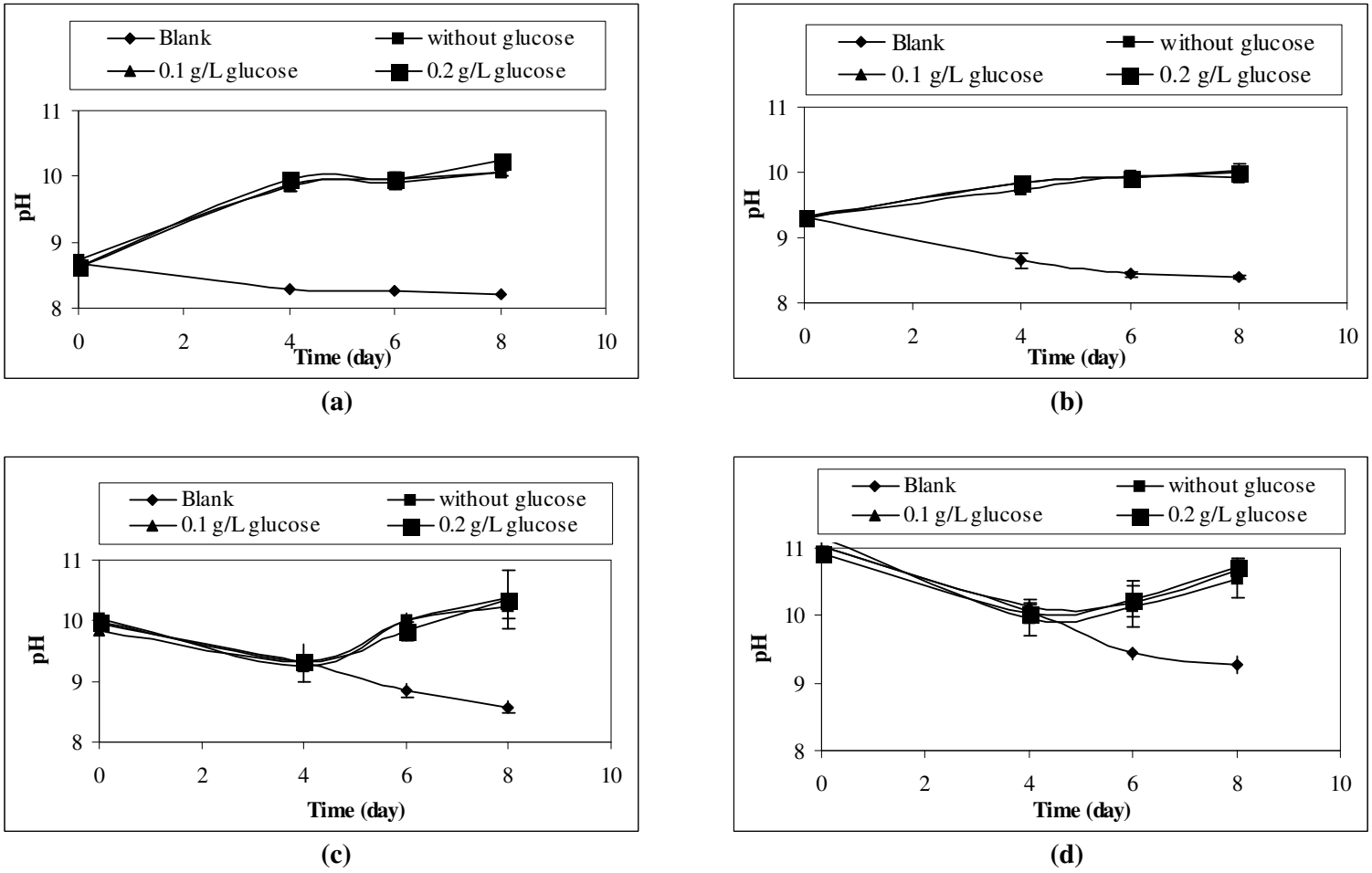


Figure 4.36. pH changes in the medium containing a) Control b) 30% RO-69, c) 60% RO-69 and d) 90% RO-69 with and without glucose.

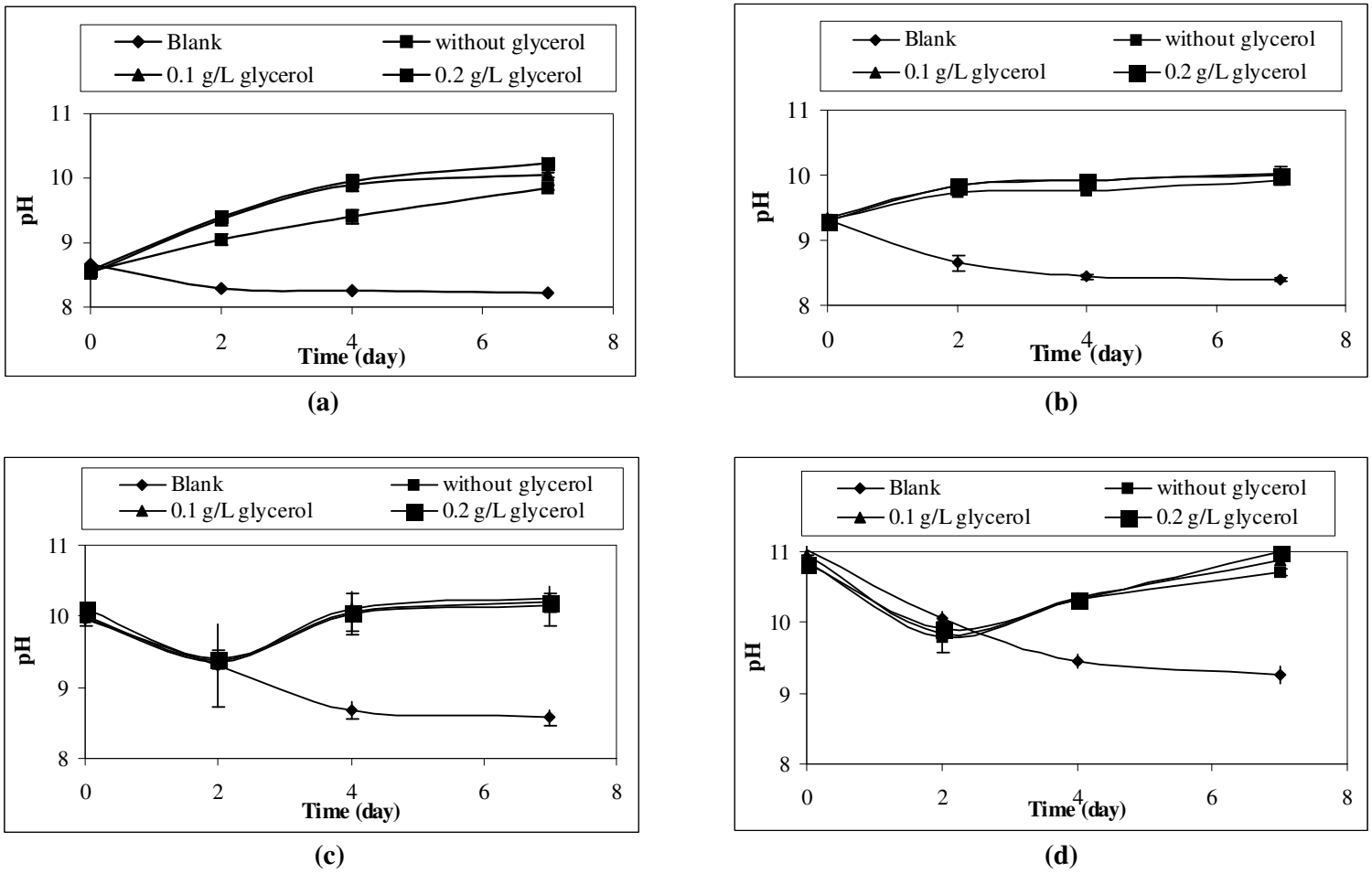


Figure 4.37. pH changes in the medium containing a) Control b) 30% RO-69, c) 60% RO-69 and d) 90% RO-69 with and without glycerol.

4.5. Reactive Yellow 37

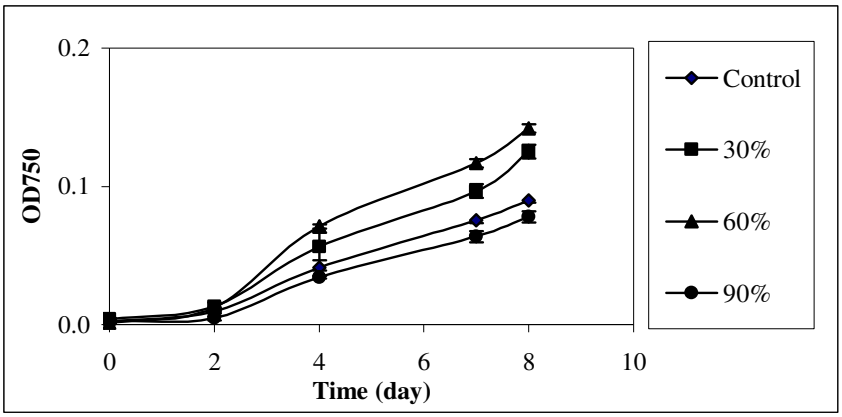
4.5.1. Growth of *Dunaliella tertiolecta* Exposed to Reactive Yellow 37 with and without Glucose

Reactive Yellow 37 (RY-37) is the second azo dye present in the dye bath recipe having sulphatoethylsulphonate as a reactive group. It also has the third highest concentration among the six azo dyes present in the dye bath recipe. To evaluate the effect of toxicity of the RY-37 with and without glucose on the growth of *D. tertiolecta* batch studies were conducted with the same dilution of DB used in glucose experiments. Growth curves of *D. tertiolecta* exposed to different dilution ratios of RY-37 with 0.1 g $L^{-1}$and 0.2 g L^{-1} glucose and without glucose were given in Figure 4.38. Error bars show the mean standard deviation between triplicates. All studied dilutions (% (v/v)) revealed a significant difference from the control ($p<0.05$).

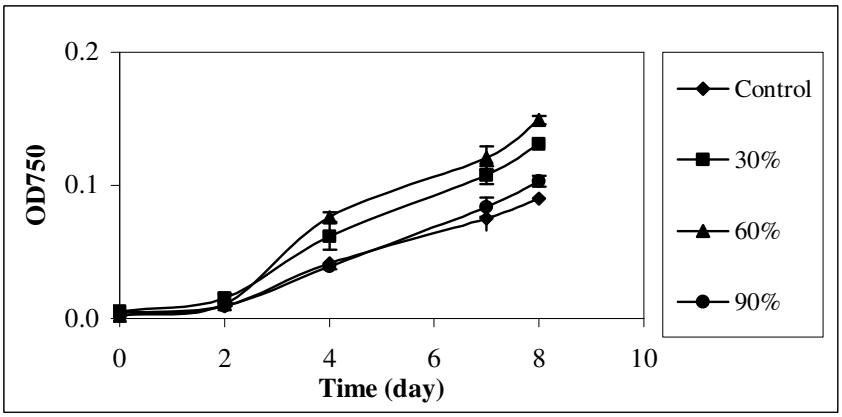
Algal growth was observed during the eight days of incubation. The response of algae exposed to RY-37 was similar to that of RB-5 and RO-69. Algal growth was promoted at low dilutions of the RY-37 and the biomass increased with the increase in number of days. A lag phase duration (four days) was observed to be almost the same at all dilutions tested (30, 60 and 90% dilutions (v/v)). On the other hand, in the exponential phase, the growth of algae exposed to RY-37 at all dilutions except 90% (v/v) was higher than that of the control (Figure 4.38 (a)). However, addition of both 0.1 g L^{-1} and 0.2 g L^{-1} glucose resulted in stimulation of the growth of algae for all dilutions compared to the single exposure of RY-37 and the control (Figure 4.32 and Table 4.7). Reduction in cell growth may result from the toxicity of dyes to algae through the inhibition of metabolic activities as stated in section 4.2.1.

While hormetic response was observed in RY-37, only stimulation was observed for RY-37 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose at all dilutions. In other words, glucose eliminated the inhibitory effects of RY-37 on the growth response of algae for all dilutions.

Calculated area under the growth curves (AUC) from optical density (OD_{750}) were tabulated in Table 4.7 together with the 8-d IC_{25} and SC_{20} and TU values. The 8-d SC_{20} values for RY-37, RY-37 with 0.1 g L^{-1} glucose and RY-37 with 0.2 g L^{-1} glucose were in



(a)



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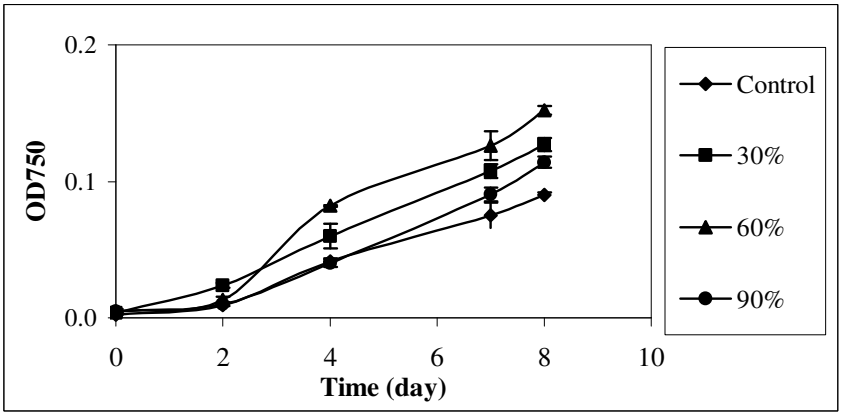


(c)

Figure 4.38. Growth curves of *D. tertiolecta* exposed to a) RY-37 B) RY-37 and 0.1 g L^{-1} glucose and c) RY-37 and 0.2 g L^{-1} glucose.

Table 4.7. Variation in optical density at 750 nm and the 8-d IC_{25} and SC_{20} of *Dunaliella tertiolecta* exposed to RY-37 in the presence and absence of glucose.

Control	% dilution (v/v)				IC_{25}[#] % dilution (v/v)	TU[*]	SC_{20} % dilution (v/v)	TU[**]
	0	30	60	90				
REACTIVE YELLOW 37								
AUC	0.20	0.28	0.36	0.17	82.30±0.20[##]	1.21	14.01±12.81 73.22±13.66	7.13 1.36
CV	7.45	6.49	6.50	2.50				
Ig (%)		-30.01	-66.74	20.96				
REACTIVE YELLOW 37 with 0.1 g L^{-1} GLUCOSE								
AUC	0.20	0.31	0.38	0.23	>100	<1	13.84±13.60 80.57±10.69	7.22 1.24
CV	7.45	5.93	5.73	2.76				
Ig (%)		-42.23	-71.71	-4.07				
REACTIVE YELLOW 37 with 0.2 g L^{-1} GLUCOSE								
AUC	0.20	0.33	0.39	0.24	>100	<1	8.84±12.88 89.52±13.06	11.30 1.11
CV	7.45	7.68	9.92	10.72				
Ig (%)		-54.41	-78.36	-8.59				

AUC = Mean values from repeated tests (n=3),
CV = Coefficient of variation
Ig (%) = % growth inhibition/stimulation; negative values show stimulation of growth with respect to the control
IC_{25} = 25% Inhibition concentration
SC_{20} = 20% Stimulation concentration
TU = Toxic Unit in terms of IC_{25}
[#] = 25% Inhibition concentration calculated from the curve fitting analysis
[##] = ± values show standard deviation
[*] = Toxic Unit in terms of IC_{25}
[**] = Toxic Unit in terms of SC_{20}

the range of 14.01-73.22%, 13.84-80.57% and 8.84-89.52% (v/v), respectively. However, inhibitory effect was observed at a higher dilution percentage when algae exposed to only RY-37. RY-37 was toxic to algae with the 8-d IC_{25} value of 82.30±0.20% (v/v). Since both concentrations of glucose eliminated the inhibitory effect of RY-37 for the studied dilutions, the 8-d IC_{25} values couldn't be calculated for RY-37 in the presence of glucose. In other words, no toxicity of RY-37 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose was detectable with an IC_{25} of >100%. When the 8-d IC_{25} and SC_{20} values of RY-37 were considered, addition of 0.1 g L^{-1} and 0.2 g L^{-1} glucose as a carbon and energy source increased the stimulatory effect of RY-37 significantly (p<0.05).

When TU values calculated on the basis of 8-d IC_{25} (TU=1.90), RY-37 was moderately toxic (TU > 1.0, Villegas-Navarro et al., 2001). However, RY-37 with 0.1 g L^{-1} and 0.2 glucose g L^{-1} was not toxic (TU<1.0 (Table 4.2)). On the other hand, TU values of RY-37, RY-37 with 0.1 g L^{-1} glucose and 0.2 g L^{-1} glucose were in the range of 1.36-7.13%, 1.24-7.22% and 1.11-11.30%, respectively when TU values were evaluated on the basis of 8-d SC_{20}. This reflects that RY-37 with 0.1 g L^{-1} and 0.2 g L^{-1} glucose and without glucose were moderately toxic (TU>1.0) at high dilutions but they were very toxic (TU≥3) at low dilutions. TU in terms of SC_{20} and IC_{25} indicating that RY-37 may cause adverse effects in an aquatic environment by stimulation of algal growth at low concentrations and an inhibition of algal growth at high concentrations. However, RY-37 with both concentration of glucose leads to only stimulatory effect on the growth of algae. These adverse effects were discussed in section 4.2.1.

4.5.2. Decolorization of Reactive Yellow 37 with *Dunaliella tertiolecta* in the Presence and Absence of Glucose

The decolorization of RY-37 with and without glucose was tested for dilutions ranging from 30% (v/v) to 90% (v/v). The cultures were monitored by considering parameters like, change in the absorbance of the prepared dye solutions, decolorization period and the effect of glucose. The UV-Vis spectra of all tested samples collected from the batch culture during a reaction period of seven days of incubation are shown in Figures from 4.39 to 4.41 to be able to determine absorbance removal at the wavelengths which RY-37 gives maximum absorbance.

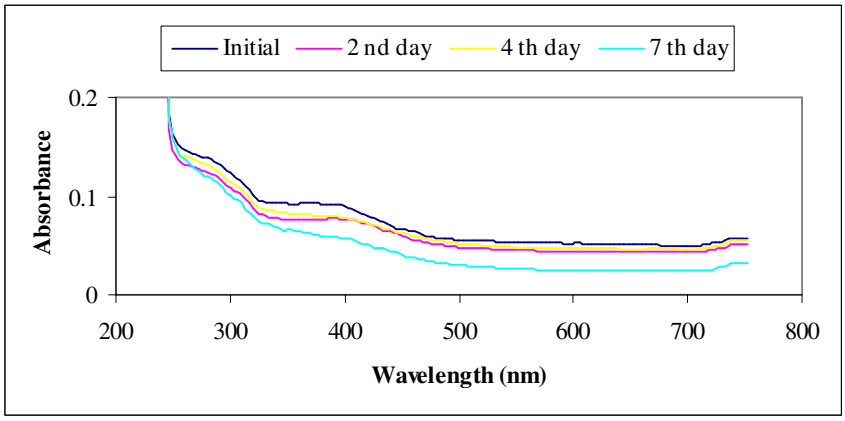
Concentration-dependent decolorization was observed. The time overlaid UV-Vis spectra of test samples of RY-37 collected during the decolorization experiments are shown in Figures from 4.39 to 4.41. While the intensity of the characteristic absorption peaks of RY-37 at 400 and 296 nm decreased drastically within four days for 30% and 60% (v/v), these peaks decreased drastically within seven days for 90% (v/v). Addition of 0.1 g L^{-1} and 0.2 g L^{-1} glucose did not change the decolorization time of RY-37 for 30%, 60% and 90% (v/v) with *D. tertiolecta* (Figures from 4.39 to 4.41 (b) and (c)).

The observations indicated that all studied dilutions of RY-37 can be removed without a co-substrate by the algae. But we expected to observe that glucose has some beneficial effects such as increasing the decolorization efficiency or decreasing the time period of spectral changes. However, there was no significant difference between decolorization of RY-37 with and without glucose for all studied dilutions.

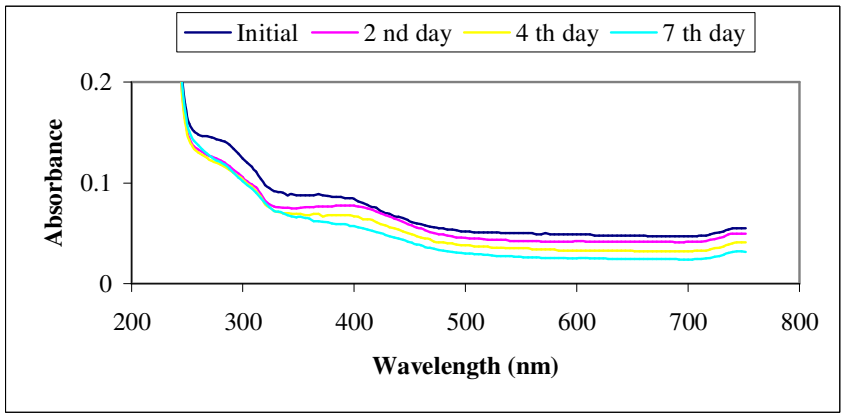
4.5.3. Color Changes during Decolorization of Reactive Yellow 37 with and without Glucose

The color values of RY-37 with and without glucose was also tested for dilutions ranging from 30% (v/v) to 90% (v/v) as a platinum cobalt (Pt-Co) unit in order to support the changes in the absorption spectra of RY-37 with *D. tertiolecta*. The variation of dye removal values are depicted in Figure 4.42 for RY-37 with and without glucose for all studied dilutions. The results showed a positive correlation between color and spectral changes.

The increase in dye concentration resulted of the reduction in color removal efficiencies. Color values decreased sharply within the first four days resulting in complete decolorization after seven days of incubation for 30% and 60% (v/v). The color removal efficiencies were 82%, 87% and 89% for 30% (v/v) dilution and 79%, 80% and 82% for 60% (v/v) of RY-37, RY-37 with 0.1 g L^{-1} glucose and RY-37 with 0.2 g L^{-1} glucose, respectively during four days. While the color removal efficiencies were 54%, 51% and 64% for 90% (v/v) of RY-37, RY-37 with 0.1 g L^{-1} glucose and RY-37 with 0.2 g L^{-1} glucose, respectively within four days, complete decolorization of 90% (v/v) of RY-37 with and without glucose was accomplished during seven days.



(a)



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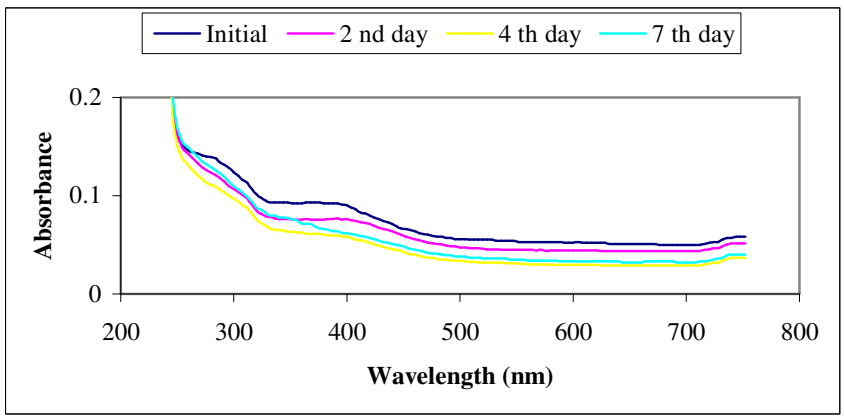
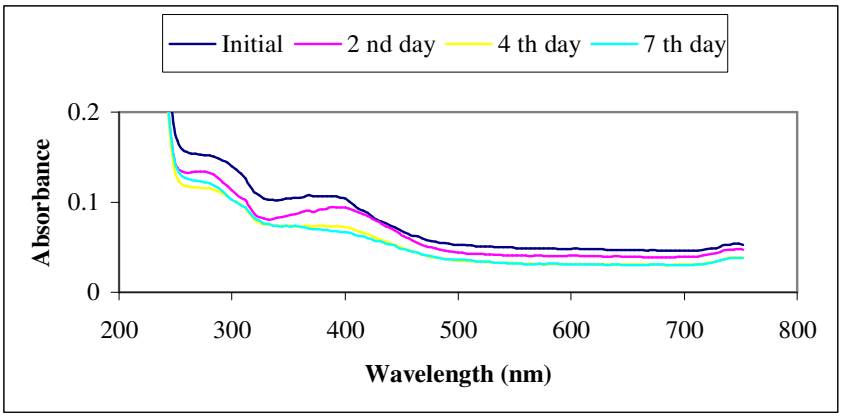
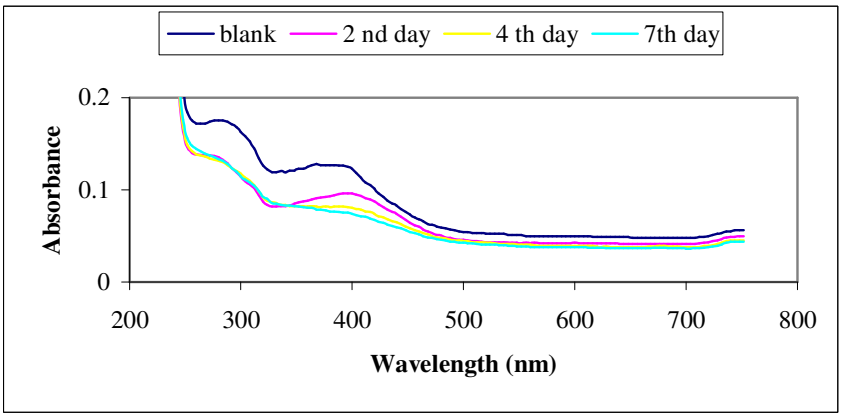


(c)

Figure 4.39. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 30% dilution (v/v) of a) RY-37, b) RY-37 and 0.1 g L^{-1} glucose and c) RY-37 and 0.2 g L^{-1} glucose.



(a)



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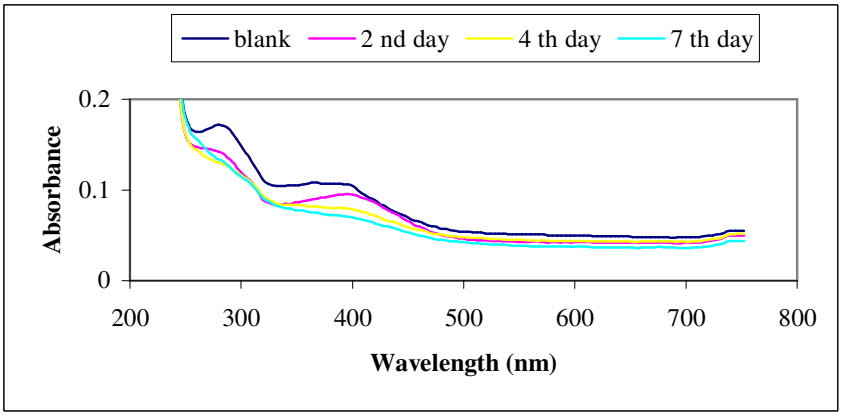
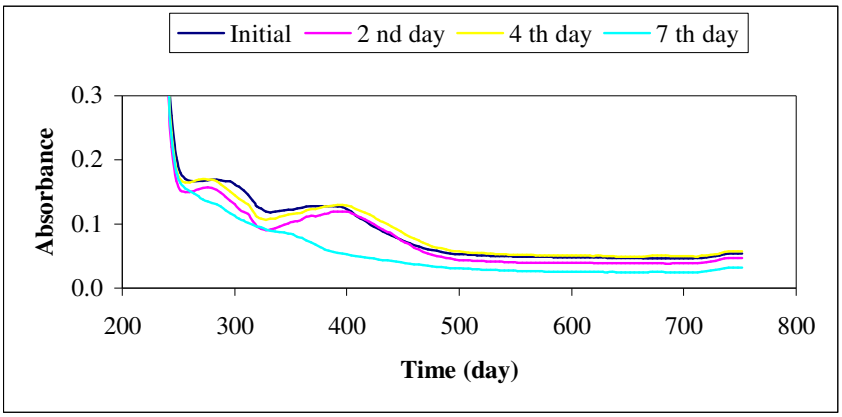
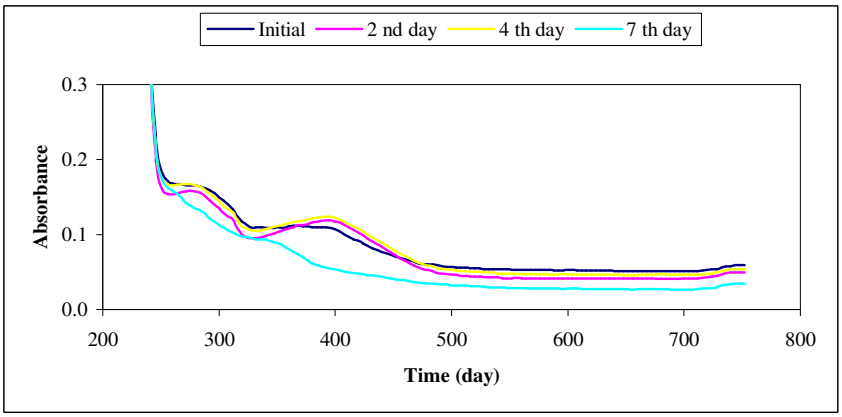


(c)

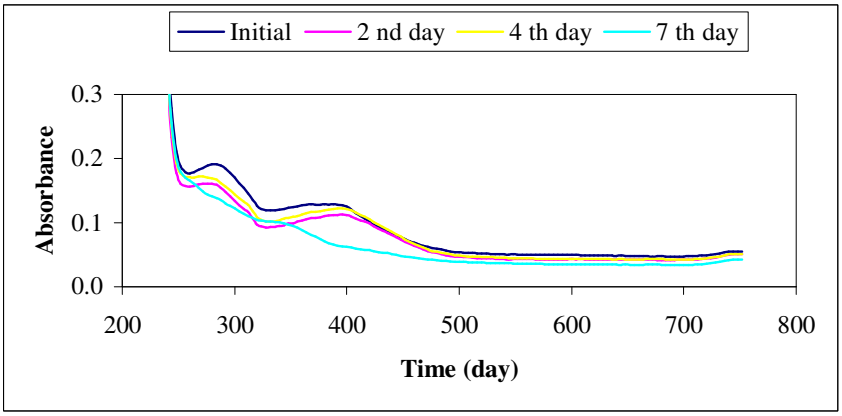
Figure 4.40. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 60% dilution (v/v) of a) RY-37, b) RY-37 and 0.1 g L^{-1} glucose and c) RY-37 and 0.2 g L^{-1} glucose.



(a)

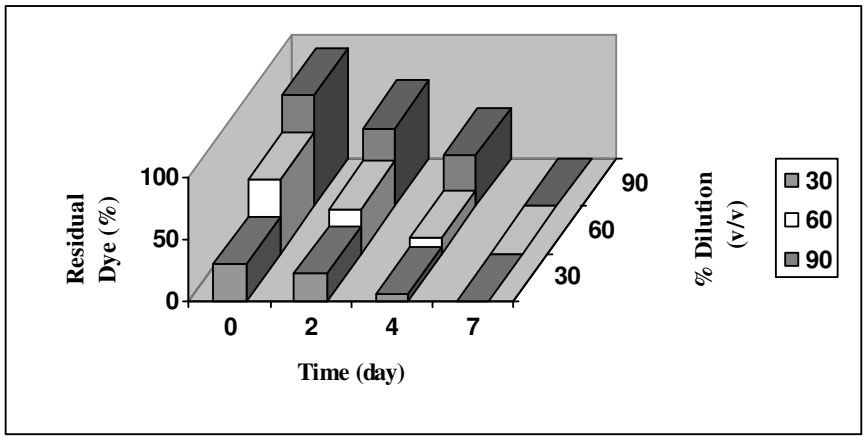


(b)

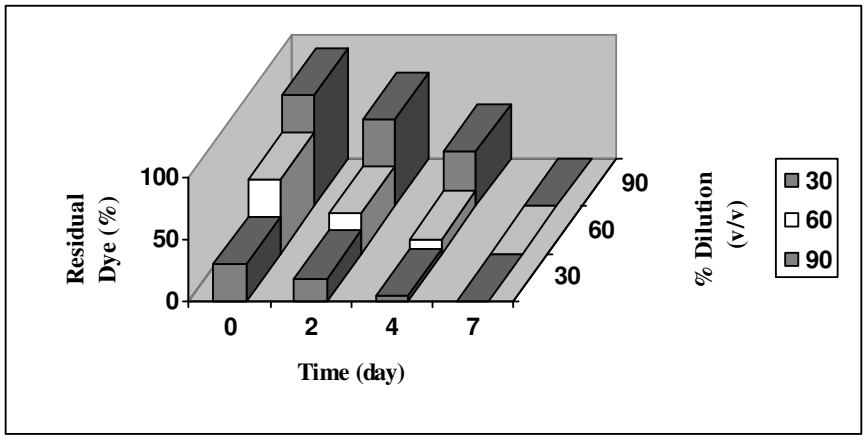


(c)

Figure 4.41. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 90% dilution (v/v) of a) RY-37, b) RY-37 and 0.1 g L^{-1} glucose and c) RY-37 and 0.2 g L^{-1} glucose.



(a)



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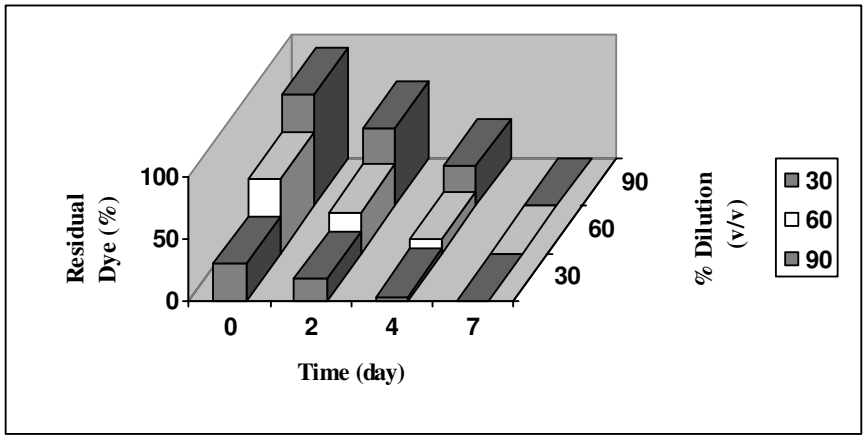


(c)

Figure 4.42. Color changes in the medium containing RO-69 a) singly b) with 0.1 g L^{-1} glucose c) with 0.2 g L^{-1} glucose.

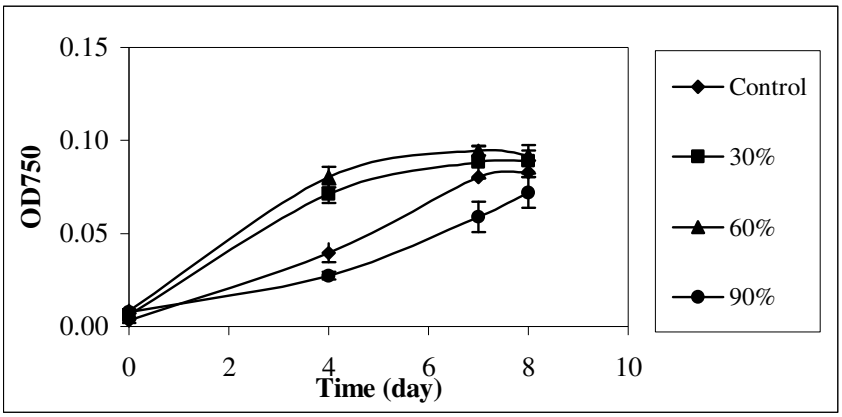
4.5.4. Growth of *Dunaliella tertiolecta* Exposed to Reactive Yellow 37 with and without Glycerol

To evaluate the effect of toxicity of the RY-37 with and without glycerol on the growth of *Dunaliella tertiolecta* batch studies were conducted with the same dilutions of DB used in glucose experiments. Growth curves of *D. tertiolecta* exposed to different dilution ratios of RY-37 with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol and without glycerol were given in Figure 4.43. Error bars show the mean standard deviation between triplicates. All studied dilutions (% (v/v)) revealed a significant difference from the control ($p<0.05$).

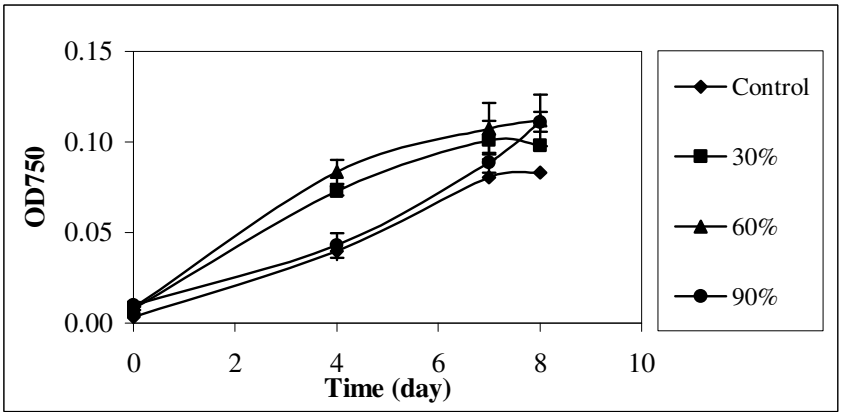
Algal growth was observed during the first eight days of incubation. A lag phase duration (four days) was observed to be almost the same for dilutions of 30% and 60% (v/v), and for 90% (v/v) and control Figure (4.43). On the other hand, in the exponential phase, the growth of algae exposed to RY-37 at all dilutions except 90% (v/v) was higher than that of the control. However, addition of both 0.1 g L^{-1} and 0.2 g L^{-1} glycerol resulted in stimulation of the growth of algae for all dilutions compared to the single exposure of RY-37 and the control (Figure 4.43 and Table 4.8). The growth of algae showed a decreasing trend with increasing dye concentration

While hormetic response was observed in RY-37, only stimulation was observed for RY-37 with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol at all dilutions. In other words, glycerol eliminated the inhibitory effects of RY-37 on the growth response of algae for all dilutions.

Calculated area under the growth curves (AUC) from optical density (OD_{750}) were tabulated in Table 4.7 together with the 8-d IC_{25} and SC_{20} and TU values. The 8-d SC_{20} values for RY-37, RY-37 with 0.1 g L^{-1} glycerol and RY-37 with 0.2 g L^{-1} glycerol were in the range of 14.01-73.22%, 10.32-84.83% and 9.19-91.74% (v/v), respectively. Inhibitory effect was observed at a higher dilution percentage. RY-37 were toxic to algae with the 8-d IC_{25} values of 82.30±0.2% (v/v). Since both concentrations of glycerol eliminated the inhibitory effect of RY-37 for the studied dilutions, the 8-d IC_{25} values couldn't be calculated for RY-37 in the presence of glycerol. In other words, no toxicity of RY-37 with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol was detectable with an IC_{25} of >100%. When the 8-d IC_{25}



(a)



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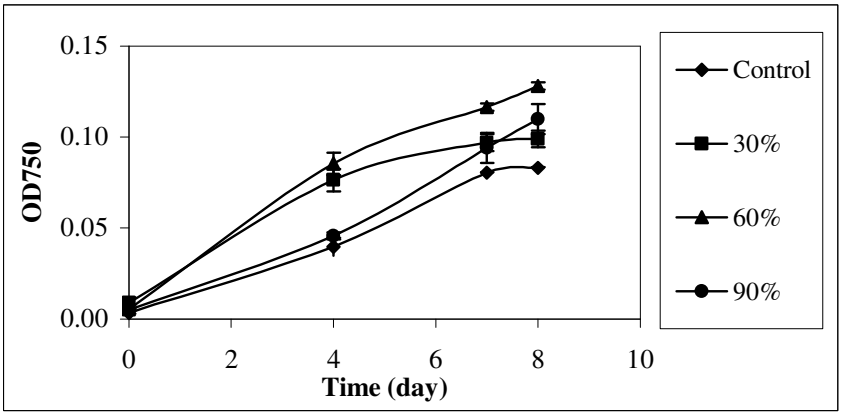


(c)

Figure 4.43. Growth curves of *D. tertiolecta* exposed to a) RY-37 B) RY-37 and 0.1 g L^{-1} glycerol and c) RY-37 and 0.2 g L^{-1} glycerol.

Table 4.8. Variation in optical density at 750 nm and the 8-d IC_{25} and SC_{20} of *Dunaliella tertiolecta* exposed to RY-37 in the presence and absence of glycerol.

Control	% dilution (v/v)				$IC_{25}^{\#}$ % dilution (v/v)	TU*	SC_{20} % dilution (v/v)	TU**
	0	30	60	90				
REACTIVE YELLOW 37								
AUC	0.20	0.28	0.36	0.17	82.3±0.20##	1.21	14.01±12.81 73.22±13.66	7.13 1.36
CV	7.45	6.49	6.50	2.50				
Ig (%)		-30.01	-66.74	20.96				
REACTIVE YELLOW 37 with 0.1 g L^{-1} GLYCEROL								
AUC	0.20	0.36	0.40	0.26	>100	<1	10.94±1.79 84.83±1.57	9.14 1.17
CV	7.45	3.14	0.86	3.22				
Ig (%)		-51.02	-68.68	-9.20				
REACTIVE YELLOW 37 with 0.2 g L^{-1} GLYCEROL								
AUC	0.20	0.35	0.44	0.27	>100	<1	9.19±0.93 91.74±3.13	10.94 1.08
CV	7.45	2.18	3.07	1.89				
Ig (%)		-47.32	-81.89	-13.71				

AUC = Mean values from repeated tests (n=3),
CV = Coefficient of variation
Ig (%) = % growth inhibition/stimulation; negative values show stimulation of growth with respect to the control
IC_{25} = 25% Inhibition concentration
SC_{20} = 20% Stimulation concentration
TU = Toxic Unit in terms of IC_{25}
# = 25% Inhibition concentration calculated from the curve fitting analysis
= ± values show standard deviation
* = Toxic Unit in terms of IC_{25}
** = Toxic Unit in terms of SC_{20}

and SC_{20} values of RY-37 were considered, addition of 0.1 g L^{-1} and 0.2 g L^{-1} glycerol as a carbon and energy source increased the stimulatory effect of RY-37 significantly ($p<0.05$).

TU value of RY-37 was 1.21 when TU values calculated on the basis of 8-d IC_{25}. Consequently, RY-37 was moderately toxic (TU > 1.0, Villegas-Navarro et al., 2001). On the other hand, TU values of RY-37, RY-37 with 0.1 g L^{-1} glycerol and 0.2 g L^{-1} glycerol were in the range of 1.36-7.13%, 1.17-9.14% and 1.08-10.94%, respectively when TU values were evaluated on the basis of 8-d SC_{20}. This reflects that RY-37 with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol and without glycerol were moderately toxic (TU>1.0) at high dilutions but they were very toxic (TU≥3) at low dilutions. TU in terms of SC_{20} and IC_{25} indicating that RY-37 may cause adverse effects in an aquatic environment by stimulation of algal growth at low concentrations and an inhibition of algal growth at high concentrations. However, RY-37 with both concentration of glycerol leads to only stimulatory effect on the growth of algae. These adverse effects were discussed in section 4.2.1.

4.5.5. Decolorization of Reactive Yellow 37 with *Dunaliella tertiolecta* in the Presence and Absence of Glycerol

The decolorization of RY-37 with and without glycerol was tested for dilutions ranging from 30% (v/v) to 90% (v/v). The cultures were monitored by considering parameters like, change in the absorbance of the prepared dye solutions, decolorization period and the effect of glycerol. The UV-Vis spectra of all tested samples collected from the batch culture during a reaction period of seven days of incubation are shown in Figures from 4.44 to 4.46 to be able to determine absorbance removal at the wavelengths which RY-37 gives maximum absorbance.

Concentration-dependent decolorization was observed. The time overlaid UV-Vis spectra of test samples of RY-37 collected during the decolorization experiments are shown in Figures from 4.44 to 4.46. While the intensity of the characteristic absorption peaks of RY-37 at 400 and 296 nm decreased drastically within four days for 30% and 60% (v/v), these peaks decreased drastically within seven days for 90% (v/v). *D. tertiolecta* decolorized RY-37 at low dilutions (30% and 60% dilutions (v/v)) resulting in

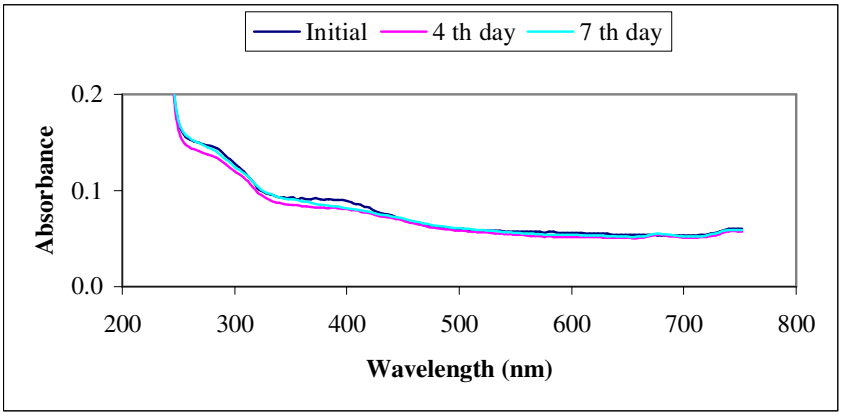
complete decolorization during four days of incubation Addition of 0.1 and 0.2 g L^{-1} glycerol did not change the decolorization time of RY-37 for 30%, 60% and 90% (v/v) with *D. tertiolecta* (Figures from 4.44 to 4.45 (b) and (c)).

The observations indicated that all studied dilutions of RY-37 can be removed without a co-substrate by the algae. This is logical because the studied concentrations of RY-37 are low compared to the other studied dyes. But we expected to observe that glycerol has some beneficial effects such as increasing the decolorization efficiency or decreasing the time period of spectral changes. However, there was no significant difference between decolorization of RY-37 with and without glycerol for the studied dilutions. This is logical because the studied concentrations of RY-37 are low compared to the other studied dyes.

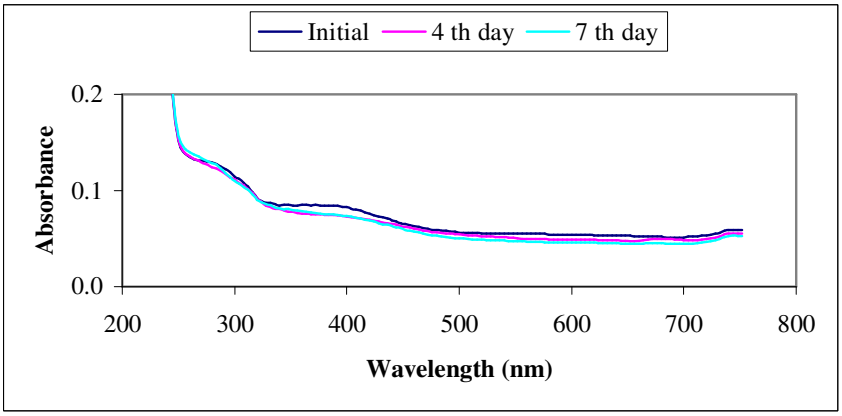
4.5.6. Color Changes during Decolorization of Reactive Yellow 37 with and without Glycerol

The color values of RY-37 with and without glycerol was also tested for dilutions ranging from 30% (v/v) to 90% (v/v) as a platinum cobalt (Pt-Co) unit in order to support the changes in the absorption spectra of RY-37 with *D. tertiolecta*. The variation of dye removal values are depicted in Figure 4.47 for RY-37 with and without glycerol for all studied dilutions. The results showed a positive correlation between color and spectral changes.

The increase in dye concentration resulted of the reduction in color removal efficiencies. Color values decreased sharply within the first four days resulting in complete decolorization during seven days of incubation for 30%, and 60% (v/v). The color removal efficiencies were 81%, 85% and 87% for 30% (v/v) and 79%, 82% and 84% for 60% (v/v) of RY-37, RY-37 with 0.1 g L^{-1} glycerol and RY-37 with 0.2 g L^{-1} glycerol, respectively during four days. While the color removal efficiencies were 41%, 46% and 47% for 90% (v/v) of RY-37, RY-37 with 0.1 g L^{-1} glycerol and RY-37 with 0.2 g L^{-1}, respectively. glycerol, respectively during four days, complete decolorization of 90% (v/v) of RY-37 with and without glycerol was accomplished during seven days.



(a)



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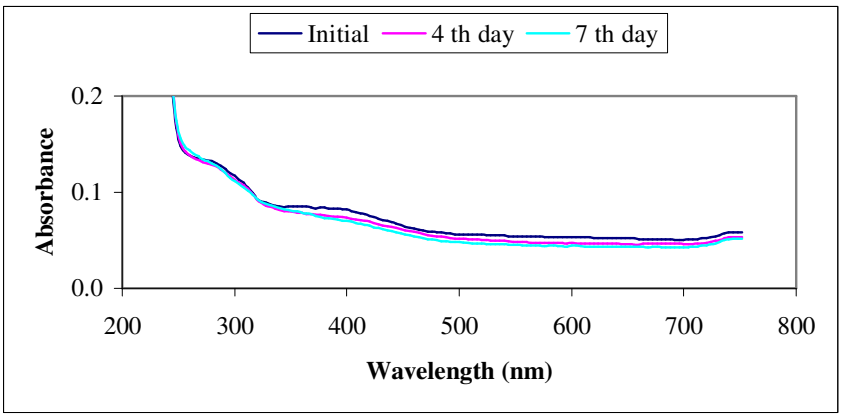
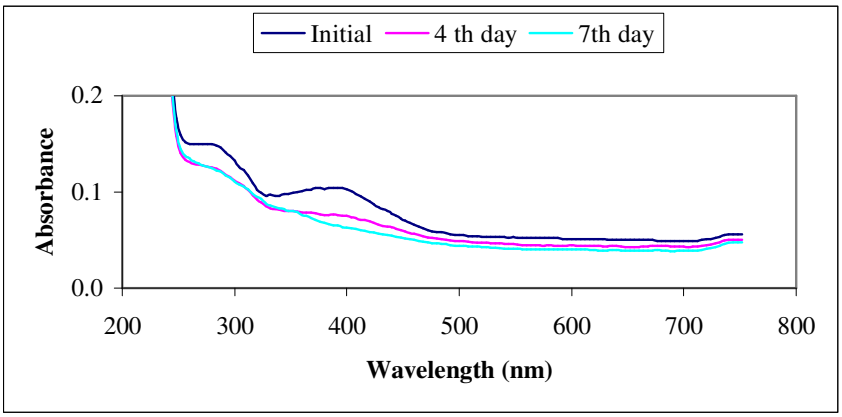
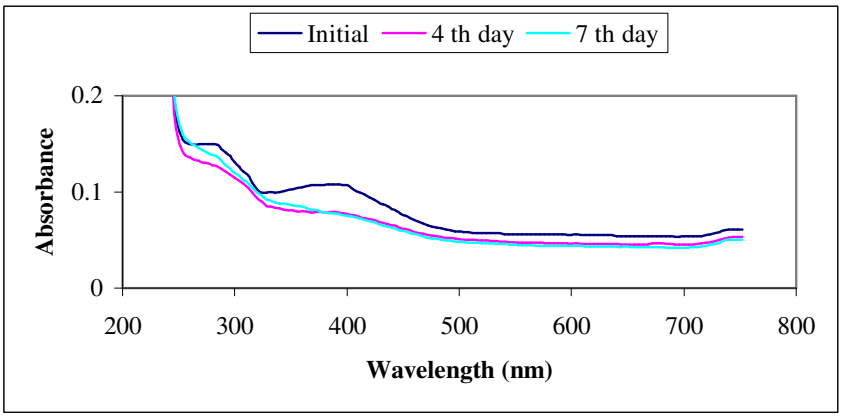


(c)

Figure 4.44. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 30% dilution (v/v) of a) RY-37, b) RY-37 and 0.1 g L^{-1} glycerol and c) RY-37 and 0.2 g L^{-1} glycerol.



(a)



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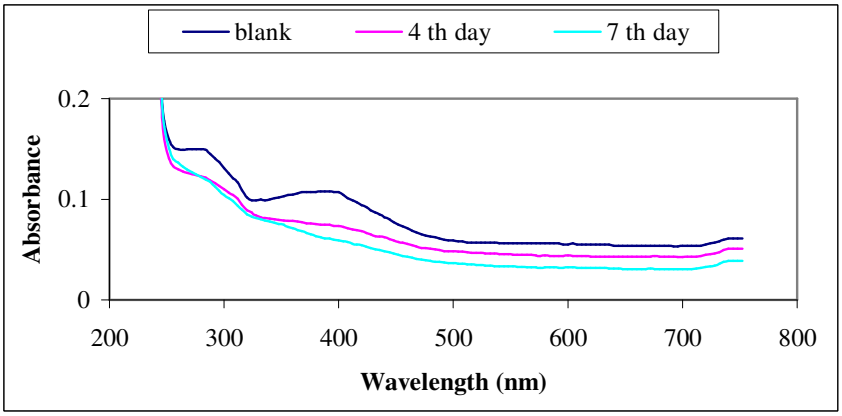
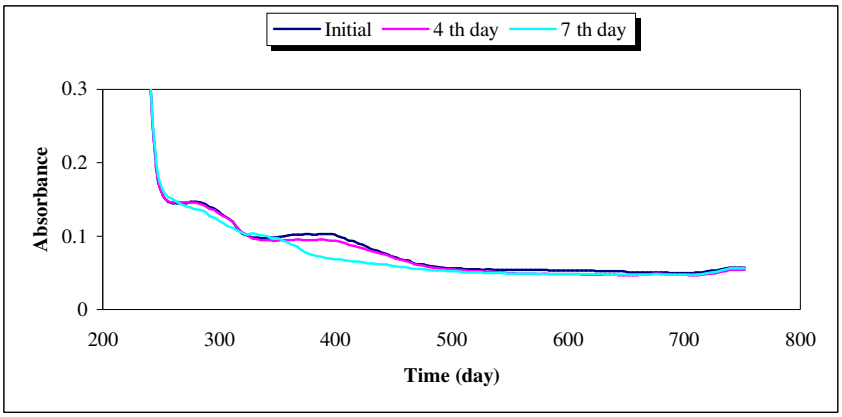
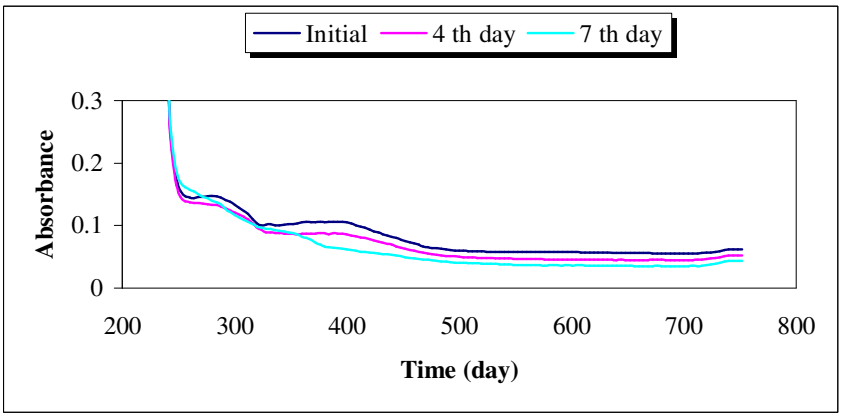


(c)

Figure 4.45. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 60% dilution (v/v) of a) RY-37, b) RY-37 and 0.1 g L^{-1} glycerol and c) RY-37 and 0.2 g L^{-1} glycerol.



(a)



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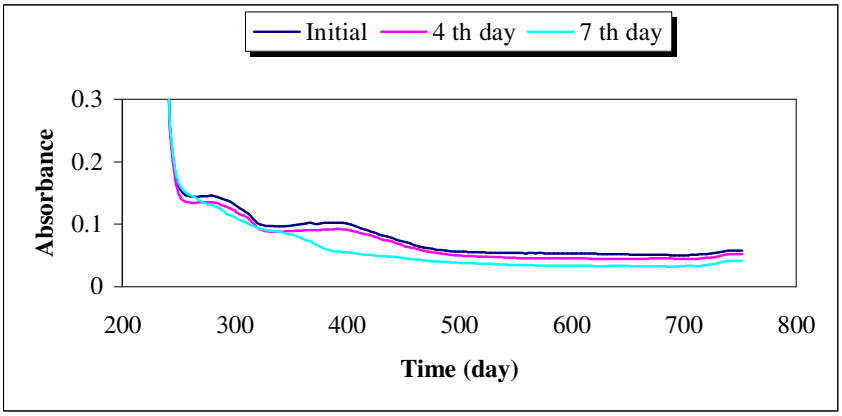
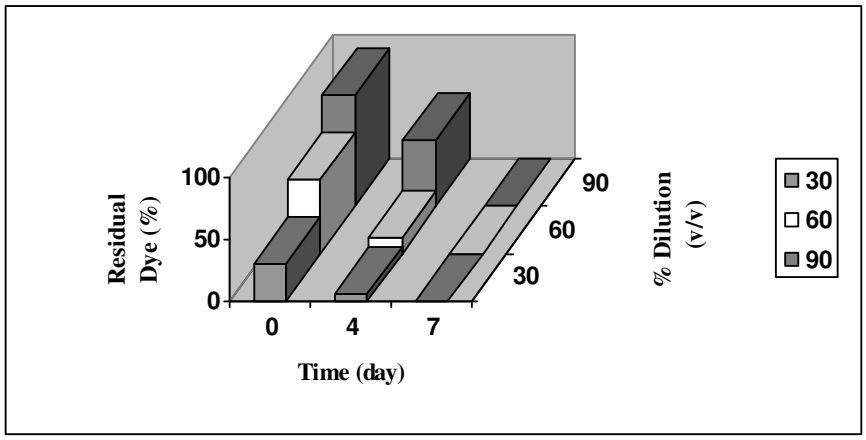
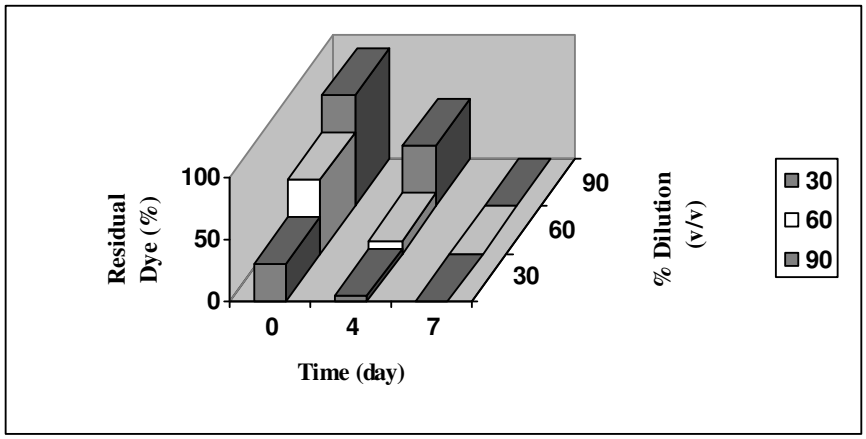


(c)

Figure 4.46. UV-Vis spectral scans from the batch cultures of *D. tertiolecta* containing 90% dilution (v/v) of a) RY-37, b) RY-37 and 0.1 g L^{-1} glycerol and c) RY-37 and 0.2 g L^{-1} glycerol.



(a)



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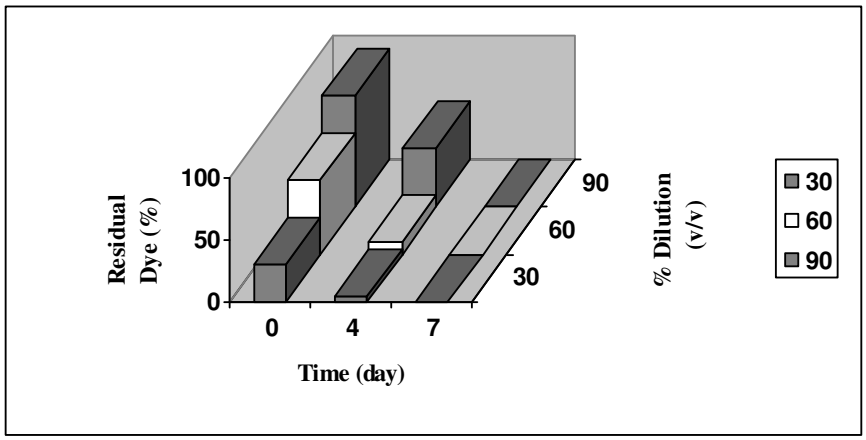

(c)

Figure 4.47. Color changes in the medium containing RY-37 a) singly b) with 0.1 g L^{-1} glycerol c) with 0.2 g L^{-1} glycerol.

4.5.7. pH Changes during Decolorization of Reactive Yellow 37 with and without Glucose and Glycerol

Changes in the pH value of the culture media during the growth period of algae exposed to RY-37 with and without glucose and glycerol are shown in Figures 4.48 and 4.49, respectively. Error bars show the mean standard deviation between triplicates. Range of pH differed notably between control and samples containing RY-37 with and without glucose and glycerol. The average pH change in blank samples was 0.36, 0.96, 1.55 and 1.97 unit for control and 30%, 60% and 90% (v/v), respectively. The pH of all the blanks decreased during eight days because of the dissolution of CO_2 and subsequent formation of H_2CO_3. Algal growth caused marked changes in the pH of the culture media, raising the low pH values (e.g., control and 30% (v/v), Figures 4.48 and 4.49 (a) and (b), respectively) and first lowering and then raising the high ones (e.g., 60 and 90% dilutions (v/v), Figures 4.48 and 4.49 (c) and (d), respectively. In fact, pH increases due to utilization of carbon dioxide and bicarbonate in photosynthesis and growth.

Within seven days all cultures exposed to RY-37 with and without glucose reached the pH range of 8.11-11.01. A sharp increase was observed in pH from 8.46 to 9.83 for the control and from 9.21 to 10.18 for RY-37 with and without glucose for 30% (v/v), then followed by almost a plateau. On the other hand, pH decreased in the test vessels containing 60% and 90% (v/v) during the first four days of growth, but it rose afterwards. While average 1.16 and 0.89 pH unit decreases were observed in the pH of the samples for 60% and 90% (v/v) with and without glucose, the average increase in pH for the same dilutions was 0.79 and 0.88 units, respectively.

The same increasing and decreasing trend was also observed for the pH of the flasks containing RY-37 with and without glycerol for the control and all dilutions (30%, 60% and 90% (v/v)). Within seven days all cultures reached the pH range of 8.51-9.96. A sharp increase was observed in pH from 8.34 to 9.77 for the control and from 8.96 to 9.82 for RY-37 with and without glycerol for 30% (v/v) then, followed by almost a plateau. On the other hand, pH decreased in the test vessels containing 60% and 90% (v/v) during the first four days of growth, but it rose afterwards. While average 0.78 and 0.95 pH unit decreases were observed in the pH of the samples for 60 and 90% (v/v) with and without glucose, the average increase in pH for the same dilutions was 0.50 and 0.84 units, respectively.

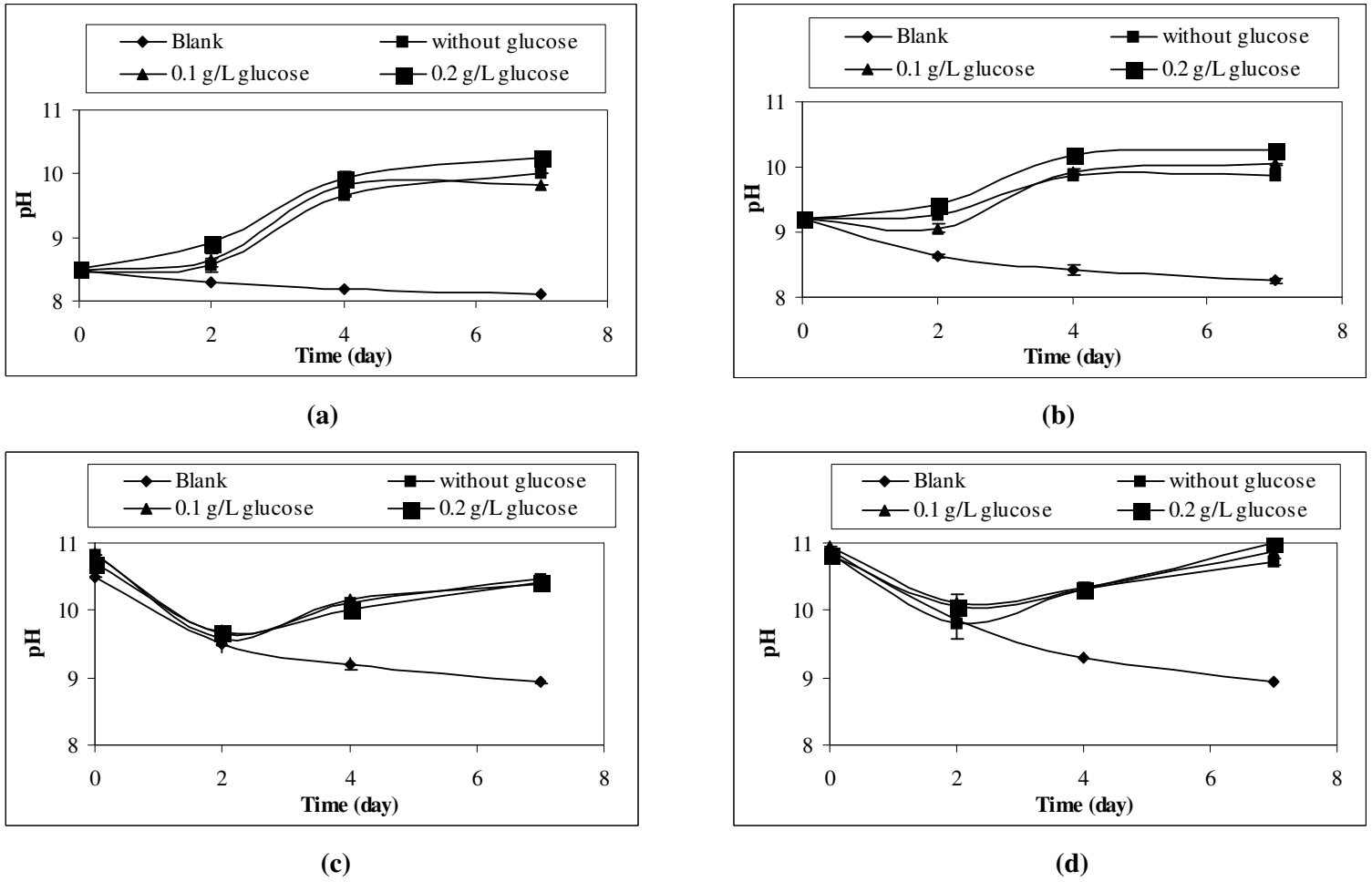

Figure 4.48. pH changes in the medium containing a) Control b) 30% RY-37, c) 60% RY-37and d) 90% RY-37with and without glucose

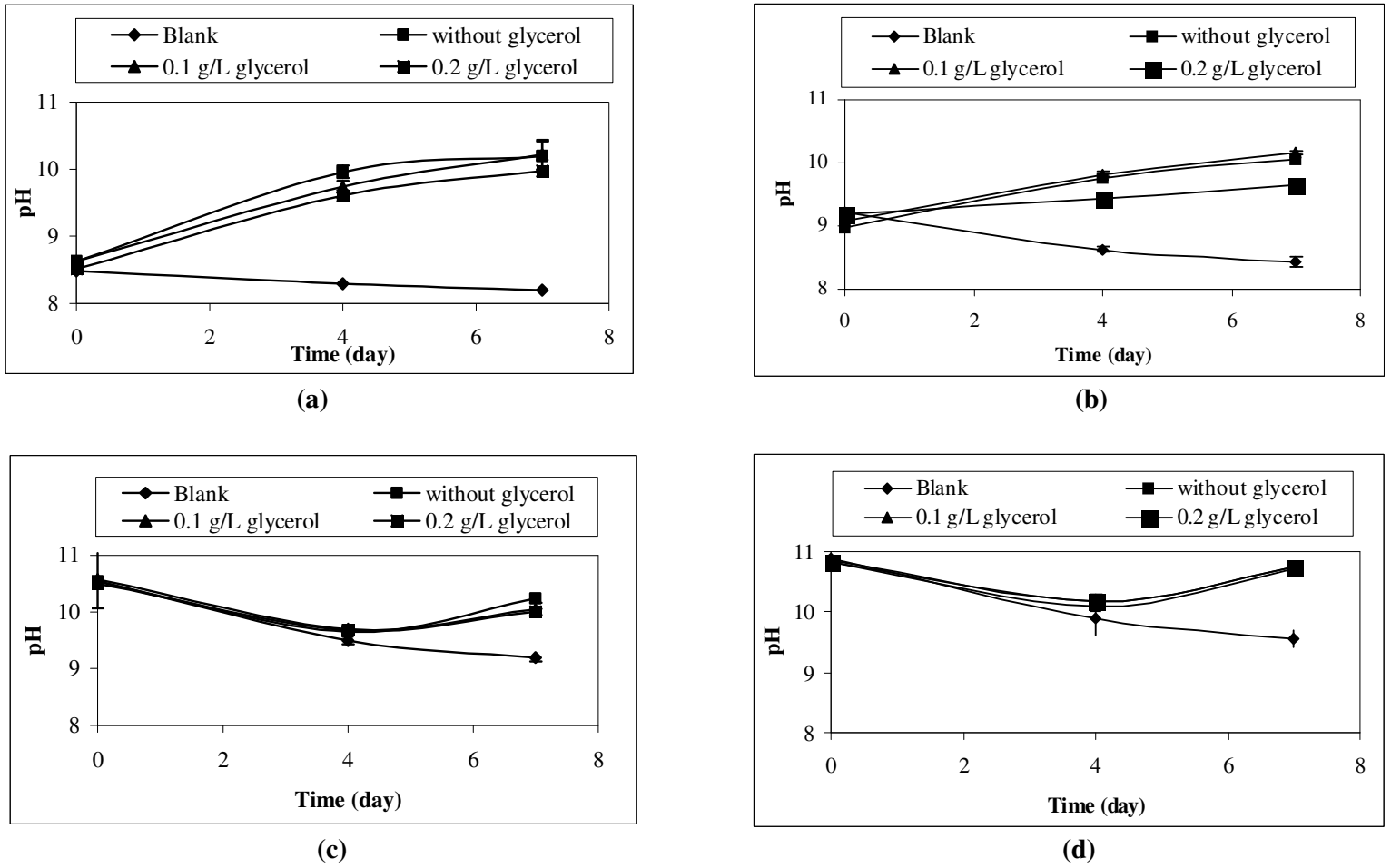

Figure 4.49. pH changes in the medium containing a) Control b) 30% RY-37, c) 60% RY-37 and d) 90% RY-37 with and without glycerol

Glucose and glycerol did not change the initial pH and pH changing trend of the medium containing RY-37, significantly. The reasons for the increasing and decreasing values of pH were stated in Section 4.2.7.

4.6. Comparision of Decolorization of Dyes by *Dunaliella tertiolecta*

The decolorization of DB, RB-5, RO-69 and RY-37 with and without glucose and glycerol was tested for dilutions ranging from 30% (v/v) to 90% (v/v). The observations indicated that low dilutions (30% and 60% dilutions (v/v)) of all dyestuffs can be removed without a co-substrate by the algae within eight days of incubation. However, complete decolorization of DB and RB-5 with and without both concentrations of glucose and glycerol for 90% (v/v) was not obtained with this culture during incubation period. In contrast to DB and RB-5, complete decolorization of RO-69 for 90% (v/v) was accomplished within six days by the addition of both concentrations of glucose and glycerol. All studied dilutions of RY-37 can be removed without a co-substrate by the algae. This is logical because the studied concentrations of RY-37 are low compared to the other studied dyes.

A gradual decrease is observed in decolorization of DB, RB-5 and RO-69 with respect to the initial concentration. The increase in dye concentration resulted in reduction of decolorization efficiencies. O'neil et al., (1999) reported that the dye concentration in the reactive dye bath effluent was observed within a narrow range of 0.1-0.2 g L^{-1}. Similarly, Sani and Banerjee, (1999) found that dyes were easily decolorized at a concentration ≤10 μM by *Kurthia* species, but color removal was reduced when dye concentration was increased to 30 μM. Although decolorization is concentration dependent, the decrease in decolorization efficiencies can be caused by the toxic effects of dyes on the blockage of active sides of azoreductase enzymes by dye molecules having different structures as stated by Işık and Sponza, 2003.

The control experiment without *D. tertiolecta* carried out using the same dilution ratios showed negligible color loss. No reaction has occurred in test vessels without cells was evident by the negligible change in absorbance at maximum wavelengths during incubation period. Similarly, Sumathi and Manju, (2000) reported that control experiments

done in the absence of fungal inoculums showed negligible color loss from the growth medium, thereby ruling out the possibility of color removal due to abiotic mechanism.

Biological reaction brought by *D. tertiolecta* lead to the changes of the absorption spectra of DB with and without glucose and glycerol. Peak disappearance was observed from the scanning of the absorbance between 200 and 750 nm for 30 and 60% (v/v) dilutions of DB, RB-5 and all studied dilutions of RO-69 and RY-37 with glucose and glycerol. This indicated that the aromatic structure of dyes used in this study was changed due to algal reactions. Nilsson et al., (2006) reported that the results from the scanning of the influents and effluents from the reactors between 200-800 nm indicate that the aromatic structure of the dyes (Reactive Blue 2 and Reactive Red 4) was changed due to microbial reactions. A slight decrease in absorbance at maximum wavelengths at 600 and 288 nm of 90 % (v/v) is likely to be due to either the abiotic mechanism or a relatively slight increase in the growth rate of algae, or both.

Spectral analysis of DB, RB-5, RO-69 and RY-37 with and without glucose and glycerol before and after algal treatment were also performed in order to understand if any intermediate or end product formation has occurred during decolorization by *D. tertiolecta*. Although the UV-Vis spectral changes show a dissappearence of peaks, there was no new peak formation. This indicates the absence of some aromatic amine and aniline which is expected to be the end product of the azo dye degradation. Some microorganisms such as wood-rotting fungi and bacteria have been reported to cause the formation of colorless and possible toxic aromatic amines (Schliephake et al., 1993). Song et al., (2003) reported that an intermediate product with λ_{max} of 340 nm was formed after decolorizaiton of Reactive Yellow X-6G, which probably was an amino derivative. Similarly, in a study reported by Khehra et al., (2006) a new peak at 320 nm was attributed to the formation of amino intermediated transformation under aerobic conditions by bacterial strains of *B. cereus, P. putida, P. fluorescence* and *S. acidaminiphila*. In addition, the appearance of a new peak at 350 nm in spectral analyses of Tectilon Yellow 2G by unacclimated algae *C. vulgaris* was attributed to the formation of aromatic amine/aniline (Acuner and Dilek, 2004). On the other hand, no aniline formation was detected at the end of the experiment with acclimated algae in the same study. They reported that aniline was probably first produced and then degraded completely by acclimated *C. vulgaris* within the experimental time period.

Decolorization seems to be growth-associated for all dyes. The results showed a positive correlation between the growth of algae and the decolorization ability measured as the absorbance removal. Decolorization activity in the culture medium increased along with the cell growth. Reduction in color removal parallel to the cell growth can be attributed to the toxicity of dyes to *D. tertiolecta* by the inhibition of metabolic activities. On the other hand, decolorization of dyes is not growth associated in the presence of a co-substrate. Mohan et al., (2002) reported that an increase in the biomass concentration indicated increased dye removal capacity, which may be attributed to the fact that the increase of biomass of algae gives more surface area for sorption of the dye molecule on the surface. In contrast to us, Chen (2002) reported that decolorization is not growth-associated, but is still a metabolic activity dependent and a basic metabolism to express azoreductase activity which must be sustained for decolorization to proceed. In case of chronic toxicity of dye to cells, a failure to preserve cellular viability leads to the occurrence of biosorption instead of decolorization.

Glucose has also been added to enhance the decolorization performance of biological systems in several studies (Haug et al., 1991; Charliell et al., 1995; Kapdan et al., 2000). On the other hand, several scientists reported that glucose inhibited the decolorizing activity (Chang et al., 1978; Knapp and Newby, 1995; White, 1995). The variability may depend on the structure of dye, characteristics of medium, experimental conditions, amount of the glucose and type of microorganisms. White (1995), reported that decolorization by bacterial strains (e.g., *P. luteola*) was often inhibited by the presence of glucose, probably due to the glucose repression that turns off expression of azoreductase. On the other hand, the yeast namely *D. polymorphus* could effectively decolorize the dye in the presence of glucose (Yang et al., 2003). Similarly, Sumathi and Manju, (2000) reported that the enhancement of color uptake with increasing concentrations of glucose may be related to increased growth rates and biomass concentration of the fungus. However, dosage of the metabolites should not exceed a certain threshold level to avoid product inhibition caused by accumulated inhibitory intermediates. The best timing of proxiding the metabolites to enhance decolorization is when cell growth had nearly stopped at the stationary phase and maximall cell was reached (Chang et al., 2001).

There is no study in the literature, which use glycerol as a co-substrate for decolorization of textile dyes. Glycerol as an additional carbon and energy source on the decolorization of DB by *D. tertiolecta* has not been studied yet and its decolorization abilities could be promising for further biotechnological applications. However, further decolorization tests are required to support these findings, by changing the dosage of glycerol.

Decolorization of the dye solution may take place in two ways: either adsorption on the microbial biomass or biodegradation of the dyes by the cells. Decolorization may be evident from the inspection of the algal growth; those adsorbing dyes will be deeply colored, whereas those causing degradation will remain colorless. Another evident note is that there is either complete removal of the major visible light absorbance peak or a significant spectral change when degradation occurs. Although dye decolorization comprises of two steps including dye adsorption on the cells and then gradual biodegradation occurred through enzymatic reactions (Wang and Yu, 1998), we didn't observe biosorption of dye when we checked adsorbed dye using the method stated in the section of materials and methods. It was possible that biodegradation of dye by *D. tertiolecta* was too fast to observe the biosorption phenomenon during the first decolorization stage as reported by Yang et al., (2005) for decolorization of RB-5 by the yeast isolates, *D. polymorphous*.

4.7. Applications of Microbial Decolorization

Pollution of water bodies arising from colored textile effluents faces increasingly stringent policies of environmental protection. Presently microbial bioremediation is regarded as a promising environmentally-friendly alternative for textile effluent treatment. In Zimbabwe, algae based waste stabilization ponds are used for wastewater treatment in most small urban areas (Dalu and Ndamba, 2003). In this study, we discussed the results of the batch culture decolorization of azo-dyes by saline water algal specie *D. tertiolecta* which is not studied before in experimental cases. The studied algae is able to decolorize azo dye at low concentrations completely, but it is able to decolorize some azo dyes at high concentrations in the presence of a co-substrate. Therefore, it seems possible to apply this treatment to the bioremediation of industrial effluents contaminated with azo dyes containing additives.

In real world applications, wastewater stabilization ponds can be used for biological treatment of textile dye-house effluents. Wastewater stabilization ponds are mainly shallow man-made basins comprising a single or several series of anaerobic, facultative or maturation ponds. Initially, treatment ponds should be filled with water from a river, lake, sea (for *D. tertiolecta*) or well depending on the used algae, so as to permit the gradual development of the algal population. Since the addition of extra carbon sources increase the cost of treatment, some of the food industry effluents containing carbohydrates may be added to supply co-substrate to the medium in the ponds. The ponds can be gradually loaded up to the design's loading rate. This gradual loading period can be changed from one to four weeks depending on the characteristics of the industrial effluent. It is important to measure the pH in the ponds and maintain it above 7 to permit the development of the algal population.

To remove the algal population from the treated wastewater is one of the shortcomings of biological decolorization of textile dye house effluents. This problem can be solved by applying biological treatment based on the combination of microalgae and macrophytes that are less expensive. Microalgal (*D. tertiolecta*) and macrophyte (Duckweeds) treatments are complementary. While firstly algae adsorb the molecules (nutrients and toxins), macrophytes eliminate the microalgal population from the water as final wastewater treatment. The microalgal biomass produced during the earlier treatment is an obstacle for reuse of the water. Since the macrophyte grows in a denser layer on the water surface, it prevents light from penetrating to the deeper layers of the bioreactor and thereby prevents microalgal growth. The macrophyte population accumulated on the water surface should remove at certain time intervals for the maintanence of treatment. The use of slow-growing grass or vegetation can be recommended to minimise the frequency of this task.

6. CONCULUSION

In this study, the decolorization of Dye Bath, and the three hydrolyzed dyes having the highest concentration in the dye bath recipe, namely RB-5, RO-69 and RY-37 by marine microalgae *Dunaliella tertiolecta*, in a batch culture was investigated and their ability to utilize the azo dyes in the presence of co-substrates namely glucose and glycerol as an additional carbon and energy sources was discussed. The color change and pH values of the culture media during the growth period of algae exposed to dyes in the presence and absence of glucose and glycerol were also investigated.

The individual and combined effects of DB, RB-5, RO-69 and RY-37 in the presence and absence of glucose and glycerol on the growth of *Dunaliella tertiolecta* were also examined. Algal growth was examined with different concentrations of glucose and glycerol. Growth response was expressed as both Ig (% inhibition/stimulation), IC_{25} (the concentration of the tested substance that decreases the growth by 25 %), TU (toxic unit) and SC_{20} (the concentration of the tested substance that increase the growth by 20 per cent).

Below is a summary of the important findings in this study:

1. Algal growth was promoted at low dilutions of the DB, RB-5, RO-69 and RY-37 and the biomass increased with the increase in number of days. A lag phase duration was observed to be almost the same at all concentrations tested (30, 60 and 90% dilutions (v/v)) for all dyes except RY-37. The growth of algae showed a decreasing trend with increasing dye concentration. However, addition of both concentrations of glucose and glycerol resulted in stimulation of the growth of algae.

2. DB has stimulatory effect on the growth of *D. tertiolecta* with the 8-d SC_{20} value of 24.94±0.37% (v/v), whereas it has inhibitory effect with the 8-d IC_{25} value of 54.90±2.22% (v/v). Addition of glucose and glycerol significantly increased the stimulatory effect of DB. Although addition 0.1 g L^{-1} and 0.2 g L^{-1} glycerol

significantly decreased the inhibitory effect of DB, both concentrations of glucose eliminated the inhibitory effect of DB for the studied dilutions. Significant difference between the 8-d IC_{25} and SC_{20} values of DB singly and in combination with glucose and glycerol was observed.

3. RB-5 has stimulatory effect on the growth of *D. tertiolecta* in the range of 8.66-51.33% (v/v) whereas it has inhibitory effect with the 8-d IC_{25} value of 62.58±6.76% (v/v). Addition of glucose and glycerol significantly increased the stimulatory effect of RB-5 at low concentrations. Both concentrations of glucose eliminated the inhibitory effect of RB-5 at high dilution. Significant difference between 8-d IC_{25} and SC_{20} values of RB-5 singly and in combination with glucose and glycerol was observed.

4. RO-69 has stimulatory effect on the growth of *D. tertiolecta* in the range of 4.91-61.27% (v/v) whereas it has inhibitory effect with the 8-d IC_{25} value of 73.68±3.53% (v/v). Within the same concentration range, RO-69 with glucose and glycerol appeared to have a stronger stimulatory effect on the growth of D. *tertiolecta* than that of RO-69 singly. Addition of both concentrations of glucose and glycerol eliminated the inhibitory effect of RO-69 at high dilution. Significant difference between the 8-d IC_{25} and SC_{20} values of RO-69 singly and in combination with glucose and glycerol was observed.

5. RY-37 has stimulatory effect on the growth of *D. tertiolecta* in 14.01-73.22% (v/v) range whereas it has inhibitory effect with the 8-d IC_{25} value of 82.30±0.20% (v/v). Addition of glucose and glycerol significantly increased the stimulatory effect of RY-37. Addition of both concentrations of glucose and glycerol eliminated the inhibitory effect of RY-37 at high dilution. Significant difference between the 8-d IC_{25} and SC_{20} values of RY-37 singly and in combination with glucose and glycerol was observed.

6. When the 8-d IC_{25} and SC_{20} values of all dyes were considered, addition of 0.1 g L^{-1} and 0.2 g L^{-1} glucose and glycerol as a carbon and energy source increased the stimulatory effect of dyes significantly ($p<0.05$) compared to single exposure of

dyes. Since 0.1 g L^{-1} and 0.2 g L^{-1} glucose eliminated the inhibitory effect of all dyes, the 8-d IC_{25} values couldn't be calculated for dyes in the presence of both concentrations of glucose. Smilarly, addition of both concentrations of glycerol eliminated the inhibitory effect of RO-69 and RY-37.

7. The 8-d IC_{25} values expressed as % dilution (v/v) indicated the sequence of decreasing toxicity as DB=RB-5>RO-69>RY-37. Toxicity of RB-5 and RO-69 to algae was higher than that of RY-37. Since the RB-5 has the highest concentration in the dye bath recipe, it is logical that the toxicity of DB is equal to RB-5.

8. When the toxicity expressed as on concentration basis (IC_{25} (µg/L)) RB-5 was the most toxic with a concentration 841±90 µg L^{-1} followed by RO-69 with a concentration 307±14 µg L^{-1} and RY-37 with a concentration of 259±1 µg L^{-1}. Since dye bath is a mixture, only % dilution value was used to express its 8-d IC_{25} values.

9. Concentration dependent reduction on the growth of algae was observed with all groups of chemical. In addition, hormetic response was observed for all studied dyes. Stimulation of algal growth at low concentrations influences the entire aquatic ecosystem by changing algal populations. If the biomass of algae becomes too high water quality may be negatively impacted. There are two main consequences of algal overproductivity. One of these is decreasing water transparency and the other one is oxygen consumption in bottom waters after settling. Decreases in water transparency may affect growth and survival of higher order, vascular aquatic plants and cause change in fish populations.

10. Inhibitory effect of synthetic dye bath and three hydrolyzed dyes decreased with the addition of glucose and glycerol. This may be due to the increase of the resistance of algae to the studied chemicals in the presence of glucose and glycerol. From these results, it is obvious that the outcome of dye-glucose and dye-glycerol interactions on algal growth is variable, and depends on the structure and concentration of dyes.

11. *D. tertiolecta* was found to be quite effective in the decolorization of all dyes. Initial dye concentratrion was found to be important in terms of both decolorization efficiency and period. The increase in dye concentration resulted in reduction in decolorization efficiencies and/or an increase in decolorization period for all dyes.

12. For DB, the observations indicated that dyestuff at low dilutions (30% and 60% (v/v)) can be removed without a co-substrate by the algae. Although addition of both concentration of glucose stimulated the growth of algae, there was no significant difference between decolorization of DB with and without glucose for the studied dilutions. However, addition of both 0.1 and 0.2 g L^{-1} glycerol has some beneficial effects such as increasing the decolorization efficiency for 30% (v/v) dilution during four days and decreasing the time period of spectral changes from six days to four days. 0.2 g L^{-1} glycerol has the same beneficial effects on the decolorization of 60% (v/v) dilution. Decolorization results of DB with and without glucose and glycerol obtained with *D. tertiolecta* culture were unsatisfactory for 90% (v/v) dilutions. Complete decolorization of 90% (v/v) dilution was not obtained with this culture during eight days of incubation.

13. RB-5 can be removed without a co-substrate by the algae at low dilutions (30% and 60% (v/v)). However, the maximum time needed for decolorization of RB-5 decreased from six days to four days with the addition of 0.2 g L^{-1} glucose. Addition of 0.1g L^{-1} and 0.2 g L^{-1} glycerol also enhanced the decolorization of RB-5 for 60% (v/v) dilution during four days. Although addition of both concentrations of glucose and glycerol was stimulated the growth of algae for all studied dilutions, complete decolorization of RB-5 with and without glucose and glycerol was not obtained for 90% (v/v) dilution with this culture during eight days of incubation.

14. RO-69 can be removed without a co-substrate by the algae at low dilutions (30% and 60% (v/v)). Addition of 0.1 and 0.2 g L^{-1} glucose decreased the decolorization time of RO-69 for 30% and 60% (v/v) dilutions with *D. tertiolecta* six days to four days However, when the batch culture was supplemented with 0.1 g L^{-1} and 0.2 g L^{-1} glucose and glycerol, complete decolorization of RO-69 for 90% (v/v) dilution

was accomplished within seven days. Although addition of both concentrations of glycerol stimulated the growth of algae at all studied dilutions, decolorization of RO-69 with 0.1 g L^{-1} and 0.2 g L^{-1} glycerol almost stayed the same for 30% and 60% (v/v) compared to single exposure of RO-69.

15. RY-37 can be removed without a co-substrate by the algae at all studied dilutions. Although addition of both concentrations of glucose and glycerol stimulated the growth of algae at all studied dilutions, there was no significant difference between decolorization of RY-37 with and without glucose and glycerol for the studied dilutions.

16. Although complete decolorization was not obtained for DB and RB-5 singly and combined with glucose and glycerol and RO-69 singly for high dilutions (90% (v/v)), complete decolorization of RY-37 was accomplished within the eight days of incubation for high dilutions. It is obvious that, at concentration beyond the IC_{25} value of dyes decolorization achievement is decreasing. The effectiveness of *D. tertiolecta* in decolorizing these three studied dyes depends on the concentration, structure and complexity of dyes.

17. Although the UV-Vis spectral changes show disappearance of peaks, there was no new peak formation in the spectrum of all studied dyes indicates the absence of some aromatic amine and aniline which is expected to be the end product of the azo dye degradation.

18. The color values of DB, RB-5, RO-69 and RY-37 with and without glucose and glycerol support the changes in the absorption spectra of dyes with *D. tertiolecta*. The results showed a positive correlation between color and spectral changes.

19. Control experiments done in the absence of algal inoculums showed negligible color loss.

20. Decolorization is also growth-associated for all dyes.. The results showed a positive correlation between the growth of algae and the decolorization ability. Reduction

in color removal parallel to the cell growth can be attributed to the toxicity of dyes to *D. tertiolecta* by the inhibition of metabolic activities. On the other hand, decolorization of dyes is not growth associated in the presence of co-substrate.

21. Glucose and glycerol did not affect the initial pH and pH changing trend of the medium containing dyes, significantly.

Although our findings could contribute to a better knowledge of the decolorization of azo dyes by *D. tertiolecta* and its decolorization ability could be promising for the future biotechnological applications, further toxicity and decolorization tests are required to support these findings. Further studies may be recommended as:

1- Investigation of decolorization of azo dyes, by acclimated algae.
2- Investigation of decolorization of azo dyes combined with higher concentration of glucose and glycerol.
3- Investigation of decolorization of azo dyes combined with different co-substrates.
4- Investigation of decolorization of azo dyes for different dilution ratios.
5- Investigation of decolorization of azo dyes at different initial pH.
6- Investigation of decolorization of azo dyes by *D. tertiolecta* to together with the change in the enzymatic activities which can be responsible for the biodegradation of azo dye.

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