Photoinactivation of *Escherichia coli* and *Enterococcus hirae* in aqueous solution

A thesis submitted in partial fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

by

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Dedicated To My Grandparents

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DECLARATION

I hereby declare that the thesis entitled **"Photoinactivation of *Escherichia coli* and *Enterococcus hirae* in aqueous solution"** is the result of investigation carried out by me at the Department of Biosciences and Bioengineeing, Indian Institute of Technology Guwahati, under the supervision of Prof. Kannan Pakshirajan and Dr. Vishal Trivedi. The work has not been submitted either in whole or in part to any other university/ institution for any degree.

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CERTIFICATE

It is certified that the work described in this thesis entitled **"Photoinactivation of *Escherichia coli* and *Enterococcus hirae* in aqueous solution"** by Ms. Madhavi Singh for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, and this work has not been submitted either in whole or in part elsewhere for a degree

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ABSTRACT

Water is essential to sustain all human related activity. However, water pollution due to different type of contamination limits its use. Among the different contaminating agents that may be present in water, coliforms poses a serious threat to the quality of water as it can lead to several diseases including cholera, dysentery, diarrhoea etc. therefore removal of these microorganisms from water prior to its use is usually carried out by a process commonly referred to as disinfection.

Disinfection is often a chemical unit operation which commonly uses chlorine, hypochlorite, chloramines, chlorine dioxide, bromine and ozone. Among these, chlorine is the most widely used disinfectant for both drinking water and tertiary treatment of wastewaters. Its popularity is due to a high oxidizing potential that provides a minimum level of residual chlorine throughout the distribution system and protects against microbial recontamination. However, it has now been realized that the disinfection by-products due to the chlorination process has both carcinogenic and mutagenic effects on mammals, and therefore, its validity has become questionable. Due to this concern, recent research is focussed towards novel disinfection methods. Compared to the existing chemical methods for disinfection, photosensitization method using photoactive compounds appears more promising due to its easy implementation and low cost. It involves the combination of three non-toxic substance, i.e. a photosensitizer, light and molecular oxygen to achieve the purpose. Although, this technique has been found successful for medical applications, it is not well studied for wastewater applications.

The present work is focused on the effect of different process parameters on inactivation of model microorganisms, viz. *Escherichia coli* and *Enterococcus hirae*, in aqueous solution using two photoactive compounds, viz. methylene blue and sodium anthraquinone-2- sulphonate. To

study the effect of parameters, experiments were carried out employing statistically valid full factorial design of experiments. Besides, the effect of municipal wastewater components on photoinactivation was examined. All these experiments were carried out under batch condition.

Initially, the effect of different parameters, viz. concentration of photosensitizer (PS), pH of the bacterial cell suspension and initial cell count, on photo-inactivation of *Escherichia coli* and *Enterococcus hirae* bacteria using methylene blue (MB) and sodium anthraquinone-2-sulphonate (SAQS) was investigated employing full factorial design of experiments. The inactivation efficiency of *E. hirae* using MB ranged between 10.81 and 48.55 %, whereas in the case of *E. coli* it ranged between10.41-46.44 %. Using SAQS, the inactivation efficiency of *E. hirae* was within 5.26-39.03 %, and in the case of *E. coli* it varied in the range 4.65-37.66 %. Statistical analysis of the photo-inactivation results in the form of analysis of variance (ANOVA) and student't' test revealed significant individual effect of these process parameters. In addition, an increase in dark incubation period with MB or SAQS enhanced the photo-inactivation efficiency against both the microorganisms. Reactive oxygen species measurement and studies of lipid peroxidation assay and protein carbonyl index in these experiments provided a clear insight into the photoinactivation mechanism involved.

The combined effect of the photosensitizers MB and SAQS for inactivating *Enterococcus hirae* and *Escherichia coli* was studied. The photoinactivation efficiency of MB and SAQS in combination and at pH 9.0 varied in the range 32.87% - 49.50% for *E. hirae* and in the range of 27.02% - 37.06% for *E. coli*. Statistical analysis of the photo-inactivation results in the form of analysis of variance (ANOVA) and student't' test revealed significant individual effect of MB on *E. hirae* inactivation but no significant effect on *E. coli*, whereas SAQS had no significant effect on both *E. hirae* and *E. coli* inactivation in this combined study.

In order to study the effect of municipal waste water components on the photoinactivation efficiency due to MB and SAQS on *E. coli* and *E. hirae*, experiments were carried out employing Placket Burman design of experiments. The photoinactivation efficiency due to MB and SAQS was less in presence of wastewater components. Statistical analysis of the photo-inactivation results in the form of analysis of variance (ANOVA) and student't' test showed that presence of synthetic wastewater components have significant effect on inactivation efficiency, with urea having maximum effect. The possible reason for this could be reaction of urea with reactive oxygen species reduces photoinactivation efficiency. In this study SAQS showed minimum inactivation against *E. coli* whereas maximum inactivation is observed with *E. hirae* and MB. Gram –ve bacteria *E. coli* has a lipopolysaccharide membrane which hinders the interaction between photosensitive dyes and the cell membrane and protects the bacteria from inactivation.

The present thesis work has successfully studied the effect of different process parameters and their interaction effect on photoinactivation of *E. coli* and *E. hirae* using photosensitive dyes MB and SAQS. Also the effect of wastewater components on photoinactivation efficiency was examined. Results showed the potential of this technology in photoinactivation of microbes in water and wastewater.

Keywords: Photoinactivation, methylene blue, sodium anthraquinone-2- sulphonate, E. coli, E. hirae, flowcytometry, synthetic wastewater

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ABBREVIATIONS AND NOTATIONS

Abbreviations

ANOVA	analysis of variance
ATP	adenosine triphosphate
BOD	biological oxygen demand
CFL	Compact Fluorescent Lamp
CO_2	carbon dioxide
CoA	acetyl-coenzyme A
COD	chemical oxygen demand
df	Degree of freedom
DCFDA	dihydrochlorofluorescin diacetate
DNPH	2,4-dinitrophenylhydrazine
F	Test static
F crit	Critical value
MS	Mean square
MB	Methylene blue
M.W	Molecular weight
P	probability 'P' value
PBS	Phosphate buffer saline
PS	Photosensitizer
TiO_2	Titanium dioxide
TCA	Tri carboxylic acid
TBARS	Thiobarbituric acid
R^2 Adj	adjusted R^2
ROS	Reactive oxygen species

Abbreviations

SAQS	Sodium anthraquinone-2- sulphonate
SS	Sum of square
UV	Ultra violet
W	Watt

Notations

°C	degree centigrade
g	gram
g	gravitational acceleration
h	hour
k_d	decay constant
D	dalton
g/l	gram per liter
min	minute
ml/min	milliliter per minute
µmol/l	Micro mol per liter
µl	Micro liter
µM	Micro mol
µJ/cm^2	Micro joules per centimeter square
R^2	regression coefficient
rpm	rotational per minute
s	second
S/N	signal-to-noise ratio
w/v	weight/volume

CHAPTER 1
INTRODUCTION

1.1 General introduction

Water is one of the most essential elements for all living organisms. Its demand has increased several folds due to increase in population and industrial activities which causes contamination of fresh water streams and depletion of groundwater level. Developing nations like India face many water related issues, such as improper management of surface runoff, contamination of surface and ground water resources, drinking water quality problems due to inefficient water treatment practices, etc. One of the major sources of water contamination, particularly in urban areas is sewage, which consists of many diverse groups of microorganisms. Due to a persistent increase in the urban population, the threat to water contamination by sewage is alarming. It is estimated that about 38,254 million litres per day (mld) of wastewater is generated in urban centres which includes class I cities and class II towns having population of more than 50,000 (Kamyotra and Bhardwaj, 2011). According to the Central Pollution Control Board (CPCB), India, there are 269 sewage treatment plants in India but only 231 are operational, which is the main cause of pollution for rivers and lakes (Kamyotra and Bhardwaj, 2011).

Municipal wastewater consists of human wastes including urine and faeces, detergents and oils (Mara, 2004). After treatment of this wastewater using technology involving mainly natural processes, it is a common practice to use such wastewater for irrigation purpose by small scale farmers in urban and peri-urban areas which causes health risks to human (Blumenthal et al., 2000; Kamyotra and Bhardwaj, 2011). Various studies have shown evidence of intestinal nematode and bacterial infections as a result of eating crops grown with sewage water. Diseases such as cholera and typhoid are also transmitted through this route

(Ferrecio et al., 1984; Shuval et al., 1986; Blumenthal et al., 2000). Hence, it becomes a necessity to treat such wastewater prior to its use.

Till date, wastewater disinfection of is accomplished by several methods including the use of physical, chemical and mechanical agents and by ultraviolet irradiation. Whereas physical agents include heat, light and sound waves, mechanical methods include screens, filters, etc. Besides UV irradiation, radiations such as electromagnetic, acoustic and particle are also used. Chemical agents used in conventional waste treatment plants include chlorine, chlorine dioxide, sodium or calcium hypochlorite, bromine and ozone (Metcalf and Eddy, 2003). However, there is some concern about the efficacy of these treatments because they either involve high cost, additional steps for removal of by products or storage. Chlorination is the most widely used method and requires more attention because it produces carcinogenic and mutagenic disinfection by-products like trihalomethanes, haloacetic acid and chlorite (Georgia's, Water Quality Report 2011).

Due to the drawbacks of conventional disinfection methods, more research is focused towards novel disinfection methods. Photosensitization currently appears to be promising under the current scenario, uses an active substance, a photosensitizer that enhances the disinfectant action of sunlight (Ergaieg and Seux, 2009). Bacterial photodynamic inactivation involves three main steps; first there is preferential accumulation of a photosensitizer in the microbial cell by different pathways (electrostatic interactions, limited diffusion, etc.). Second, targeted illumination at an appropriate wavelength induces photochemical activation of oxygen into its excited state (singlet oxygen, $^1O_2$) which is a highly reactive species. Third, oxygen reacts with various neighbouring macromolecule targets in the bacteria resulting in lethal damage to the cell (Malik et.al, 1990; Ergaieg and Seux, 2009). Schematic representation of the mechanism involved in photo-inactivation is shown in Figure 1.

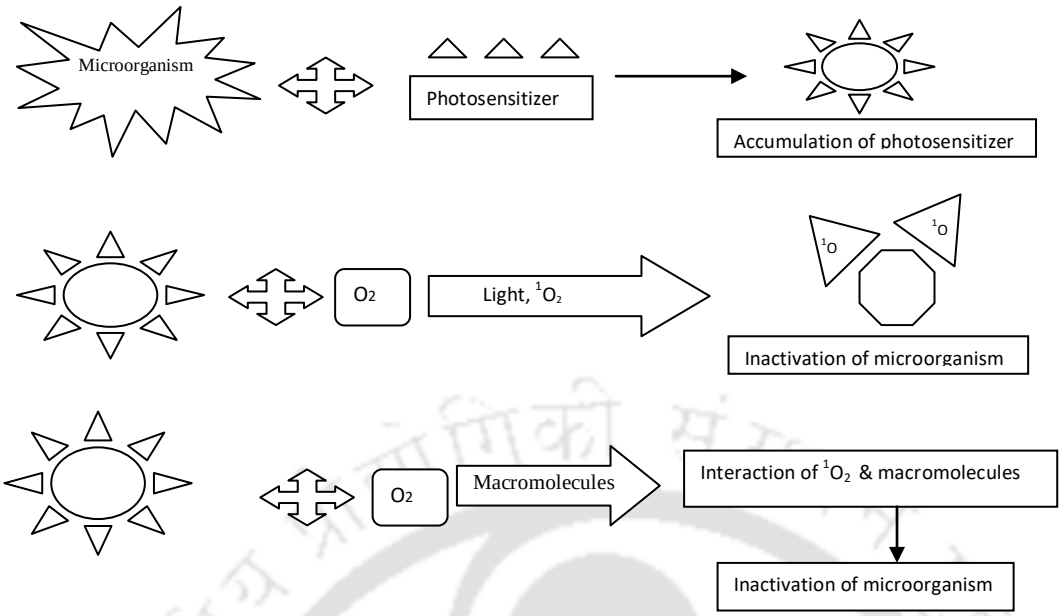


Fig. 1.1 Steps involved in inactivation of microorganisms by photosensitizers (a) accumulation of photosensitizer (b) reaction with singlet oxygen (c) reaction with singlet oxygen via macromolecule

Two oxidative mechanisms are considered to be principally implicated in the photodamage of cells. Type I mechanism involves hydrogen atom abstraction or electron transfer between the excited sensitizer and a substrate, yielding free radicals. These radicals can react with oxygen to form an active oxygen species, e.g. superoxide radical anion. In Type II mechanism, singlet oxygen is generated via an energy transfer process during a collision of the excited sensitizer with triplet oxygen (DeRosa and Crutchley, 2002; Kuznetsova et al., 2007).

Other than singlet oxygen species hydroxyl radicals and superoxide ions are also produced for disinfection. Hydroxyl are known to be very reactive, possibly more reactive than singlet oxygen especially in lipids, where singlet oxygen exhibits a long lifetime and is thought to be less reactive (Chen et al., 2011).

In order that photosensitive compounds could be efficiently used for inactivating microorganisms in water, besides understanding their mechanism of action, effect of the nature and concentration of these compounds, pH, microorganisms type on the disinfection efficiency need to be examined in detail.

The present research work therefore investigated all these aspects using *Escherichia coli* and *Enterococcus hirae* as the model organisms for their inactivation in aqueous solution by two different class of photoactive compounds, viz. methylene blue and sodium anthraquinone-2-sulphonate.

1.2 Aim and Objectives

The main aim of the study is photo-inactivation of *Escherichia coli* and *Enterococcus hirae* in aqueous solution.

To fulfill the above aim following investigations are carried out

1. Effect of parameters viz. type and concentration of dye, pH, dilution, type of light source and dark incubation time on photo-inactivation of *E. coli* and *E. hirae*
2. Combined effect of mythelene blue (MB) and sodium anthraquinone-2-sulphonate (SAQS) on photo-inactivation of *E. coli* and *E. hirae*
3. Photo-inactivation of *E. coli* and *E. hirae* in synthetic wastewater

1.3 Organization of the thesis

The present work has been divided into five chapters. The first chapter contains general introduction of photo inactivation of microorganisms, aims and objective of the work and organization of the thesis. In the chapter 2 detailed literatures that supports the present work is included. Chapter 3 contain materials and methods used in this work. That chapter provides

technical details about photo inactivation of *E. coli* and *E. hirae* using photosensitizers (PS), experimental designs used for the study, bacterial cell quantification methods and details instruments used. Chapter 4 consists of the results and discussion. This chapter starts with results obtained for photoinactivation of the selected microorganisms using single PS at different pH, dilution, concentration of PS. Followed by results of studies on ROS generation and lipid peroxidation is reported. The combined effect of PS on photo inactivation of *E. coli* and *E. hirae* is also included in this chapter. Finally the result of photo inactivation of *E. coli* and *E. hirae* in synthetic wastewater and effect of wastewater composition on photo inactivation efficiency is reported. Chapter 5 draws summery and appropriate conclusions based on the previous results and discussion. This chapter also provides some useful recommendations for future research in the relevant area.

CHAPTER 2
LITERATURE REVIEW

2.1 Water Pollution

Water pollution is a major global problem which requires ongoing evaluation and revision of water resource policy at all levels (international down to individual aquifers and wells). It has been suggested that water pollution is the leading worldwide cause of deaths and diseases (Michael, 2010) and that it accounts for the deaths of more than 14,000 people daily (Dunlap and Jorgenson, 2012). An estimated 580 people in India die of water pollution related illness every day (Hardoy and Satterthwaite, 2014). In addition to the acute problems of water pollution in developing countries, developed countries also continue to struggle with pollution problems. For example, in the most recent national report on water quality in the United States, 44 percent of assessed stream miles, 64 percent of assessed lake acres, and 30 percent of assessed bays and estuarine square miles were classified as polluted (Gleick and Ajami, 2014).

Water is typically referred to as polluted when it is impaired by anthropogenic contaminants and either does not support a human use, such as drinking water, or undergoes a marked shift in its ability to support its constituent biotic communities, such as fish. Any modifications or change in the chemical, physical and biological properties of water that can cause any harmful consequences on living things and the environment is known as water pollution. Water pollution is very often caused by human activities (Greenstone and Hanna, 2014; Wasi et al., 2013).

2.1.1 Sources of Pollution

Point sources

Point source pollution is defined by the U.S. Environmental Protection Agency (EPA) as "any single identifiable source of pollution from which pollutants are discharged, such as a pipe or ditch" (EPA, 1990). Examples of point sources include discharges from wastewater treatment plants, operational wastes from industries and combined sewer outfalls.

Non Point Sources

Pollution caused by nonpoint sources refers to the contamination that does not originate from a single source. Nonpoint source pollution is the cumulative effect of small contaminants gathered in large area. We all contribute to non-point source pollution when we improperly use or dispose of fertilizers, pesticides, oils, grease, pet or animal wastes, and trash. In many communities, these pollutants are transported to local waterways via storm drains.

Examples of non-point sources of pollution includes sediments from construction, forestry operations and agricultural lands, bacteria and microorganisms from failing septic systems and pet wastes, nutrients (from fertilizers and yard debris) and pesticides from agricultural areas, golf courses, athletic fields and residential yards, oil, grease, antifreeze, and metals washed from roads, parking lots and driveways, toxic chemicals and cleaners that were not disposed of properly, litter thrown onto streets, sidewalks and beaches, or directly into the water by individuals (Gupta et al., 2012).

Among the different pollutants that may be present in water, microorganisms poses a serious threat to human health. Though some microorganisms are helpful, a large number of them are harmful and can lead to several diseases including cholera, dysentery, diarrhoea etc. (Wasi et al., 2013).

2.2 Microorganisms in water

Water microbiology is concerned with the microorganisms that live in water, or can be transported from one habitat to another by water. Water can support the growth of many types of microorganisms. Disease-causing microorganisms spread to aquatic wildlife and plants and possibly even to human beings. Microorganisms can also reduce the amount of oxygen in the water, thereby hindering the water's ability to support aquatic animal and plant life (Greenstone and Hanna, 2014).

Microorganisms, including bacteria, viruses and parasites, can originate from the following sources:

Improperly treated sewage: Contamination of drinking water sources by sewage can occur from raw sewage overflow, septic tanks, leaking sewer lines, land application of sludge and partially treated waste water. Sewage itself is a complex mixture and can contain many types of contaminants including pathogenic microorganisms (Hardoy and Satterthwaite, 2014).

Runoff from animal wastes: More than 150 microbial pathogens have been identified from all animal species that can be transmitted to humans by various routes (USDA, 1992; USEPA, 1998). Pathogens can be transmitted from animals to humans when manure is used as a fertilizer for food crops eaten raw and by storm water runoff from manured surface-to-surface waters or by its percolation to ground water. Industrial sources such as slaughter houses, food and paper processing plants and some landfills are also contribute to microbial contamination of water bodies (Liang et al., 2014).

2.2.1 Classes of microorganisms

The greatest microbial risks are associated with ingestion of water that is contaminated with

human or animal feces (WHO). Major public health problem in developing countries are acute microbial diarrheal diseases. People affected by diarrheal diseases are those with the lowest financial resources and poorest hygienic facilities. Children under five, primarily in Asian and African countries, are the most affected by microbial diseases transmitted through water (seas et al., 2000). Microbial waterborne diseases also affect developed countries. In the USA, it has been estimated that each year 560,000 people suffer from severe waterborne diseases, and 7.1 million suffer from a mild to moderate infections, resulting in estimated 12,000 deaths a year (Medema et al, 2003). Fig 2.1 gives a detailed account of disease causing microorganisms under different groups such as Bacteria, Viruses, Protozoa, Parasites and Algae.

2.2.2 Indicator Microorganisms

Fresh water bodies which serve as drinking water source when gets contaminated by wastewater discharges or storm runoff shows evidences of microbial contamination. To look for specific pathogens for cholera, typhoid, hepatitis etc. is a tedious job because they are rare, hard to isolate, takes long time to be identified and their absence does not mean that the water is potable. So we look for indicator microorganisms whose presence indicates the possible presence of pathogenic organisms. Criteria for ideal indicator oraganisms are should be non-pathogenic, detectable by means of easy, rapid and inexpensive methods, Present in greater number than the pathogen, member of the intestinal micro flora of warm-blooded animals. None of the indicator organisms that are currently in use fit all of these criteria perfectly, however, when cost is considered, use of indicators becomes necessary. Common indicator microorganisms include total coliforms, fecal coliforms, *E. coli* and *Enterococci*.

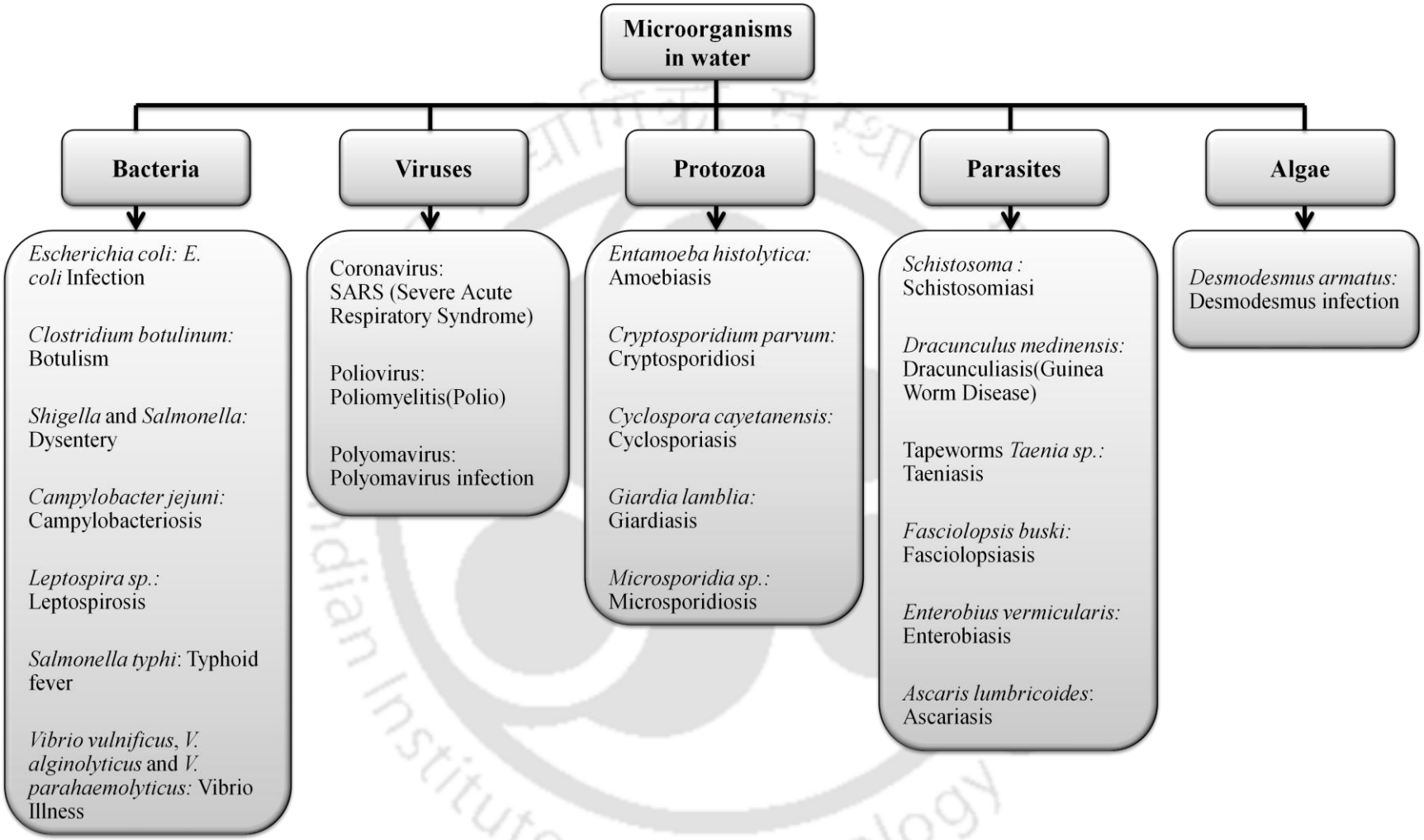


Fig. 2.1 Some common microorganisms in water and diseases caused by them (Mara et al., 2004; Petrini et al., 2006; Dziuban et al., 2006; Nwachcuku et al, 2005)

2.2.3 Test for Viability

It is essential to determine the number of viable microorganisms present in a given sample. A variety of methods has been developed for the enumeration of viable microbes. These methods measure cell numbers, cell mass, or cell constituents that are proportional to cell number. They can be grouped in direct and indirect methods.

2.2.3.1 Direct methods

Direct methods generally use instrumental techniques such as flowcytometry, fluorescence microscopy, fluorescein diacetate hydrolysis.

Flow cytometry provides a rapid and reliable method to quantify viable cells in a cell suspension. To assess cell viability dye exclusion method can be used. A variety of dyes are excluded by live cells due to the presence of intact membranes but could easily penetrate the damaged, permeable membranes of non-viable cells. Propidium iodide (PI) is one such dye which is excluded by viable cells. It gets bound to double stranded DNA by intercalating between base pairs and is excited at 488 nm and emits at a maximum wavelength of 617 nm. These spectral characteristics makes PI suitable to be used in combination with other fluorochromes excited at 488 nm such as fluorescein isothiocyanate (FITC) and phycoerythrin (PE).

For fluorescence microscopy two colour fluorescence staining of nucleic acids of both live and dead cells using DMAO a green fluorescent dye, which stains both live and dead bacteria with both intact and damaged cell membranes and EtD-III a red fluorescent nucleic acid dye which stains only dead bacteria with damaged cell membranes. The optimal concentrations of the dyes depend on the bacteria types.

Fluorescein diacetate (FDA) hydrolysis assay is used to measure enzyme activity of the microbes in a sample. If the enzymatic activity is high a fluorescent green colour is produced which could be quantified using a spectrofluorometer. This method is often used to measure microbial activity in the soil and compost samples. If the microbes have the ability to cleave the fluorescein an accurate reading of the activity is impossible (Green et al., 2006).

2.2.3.2 Indirect methods

The Most probable number (MPN) method by concept is similar to the fraction negative method of D-value determination. Here, the growth of organisms is supported by nutrient broth or any other growth media which turns turbid and the pattern of growth vs. no-growth provides the information about sampling error. This information is particularly useful at low numbers of organisms. To increase the accuracy the inoculum could be diluted and the recoveries of all the tubes in the dilution series could be compared. This is the basis of the MPN method (Sutton, 2010).

The plate count method is most commonly used method, carried out by diluting a sample with sterile saline or phosphate buffer until the bacterial suspension is diluted enough to count the cells accurately i.e., the plates in the series should have between 30 and 300 colonies. The assumption is that each viable bacterial cell is separate from all others and will develop into a single discrete colony (CFU). As the exact number of bacteria is usually unknown, a wide series of dilutions (e.g., 10^{-4} to 10^{-10}) are normally plated. To achieve greater accuracy plating is done in duplicates or triplicates of each dilution (APHA, 1998). Fig 2.2 gives a detailed description of MPN, plate count and MTT assay along with their advantages and disadvantages.

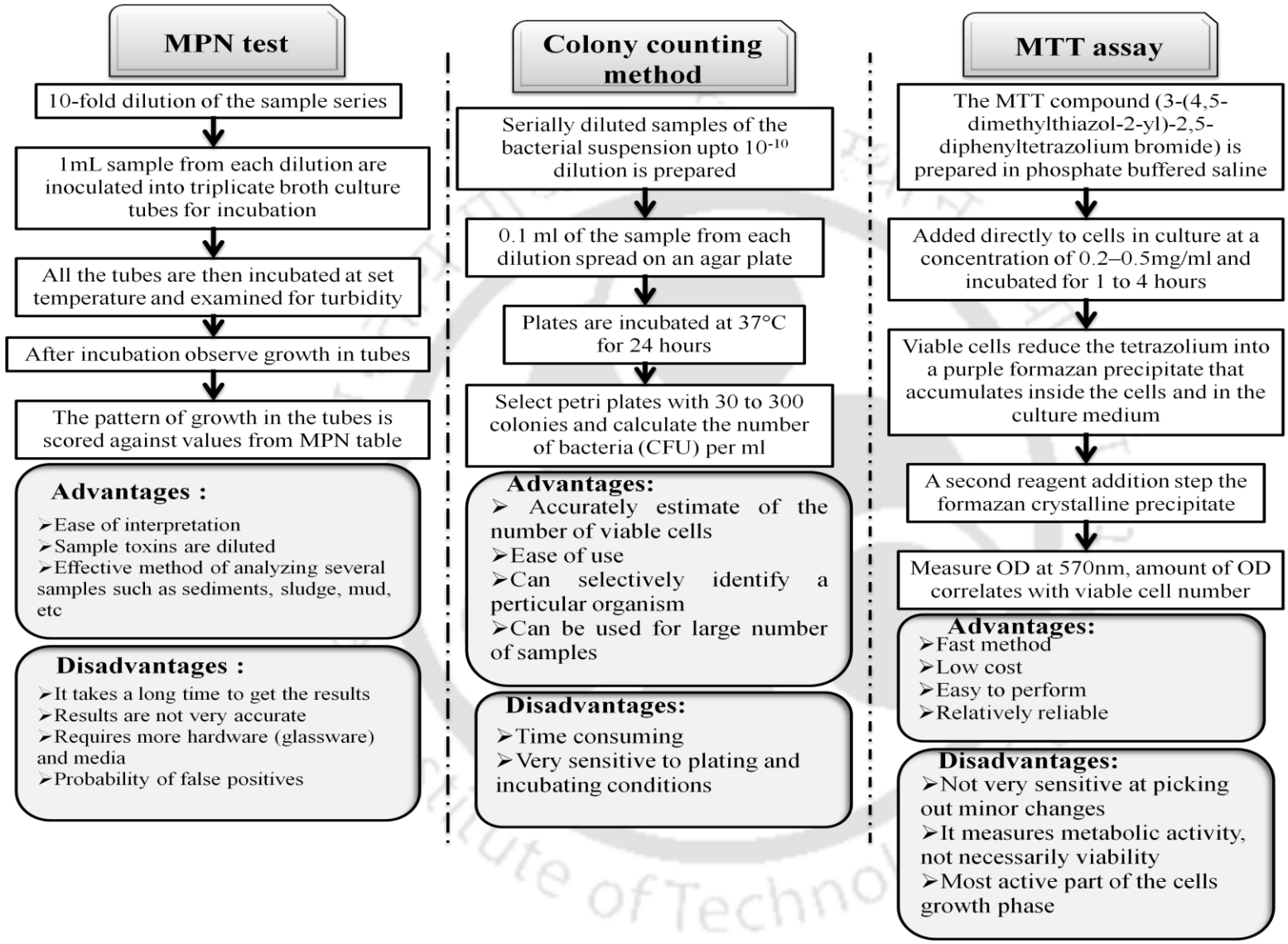


Fig. 2.2 Description of methods used for MPN, plate count and MTT assay (Sutton, 2010; APHA, 1998 and Buch et al., 2012)

MTT assay is a colorimetric assay which measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by succinate dehydrogenase of mitochondria. MTT is capable of entering the cell and passes to the mitochondria where it is reduced to a dark purple coloured product, formazan. The cells are now solubilised using an organic solvent (e.g. isopropanol) and the released formazan is measured spectrophotometrically. MTT reduction is only possible in metabolically active cells so the level of activity is directly related to the viability of cells (Buch et al., 2012).

2.3 Water disinfection

Disinfection is the process of elimination of most pathogenic microorganisms (excluding bacterial spores) on inanimate objects. It could be achieved by physical or chemical methods. Chemicals used in disinfection are called disinfectants. Different disinfectants have different target ranges, not all disinfectants can kill all microorganisms. Sterilization and disinfection are not synonymous because sterilization is an absolute condition while disinfection is not.

2.3.1 Physical disinfectants

Physical disinfectants which could be used are heat, light (radiation) and waves.

Heat: Heat is considered to be most reliable method of disinfection, for example, boiling of water to kill the disease causing microorganisms. It acts by oxidation or denaturation and coagulation of proteins. Factors affecting disinfection by heat are:

Radiation: For disinfection ionizing and non-ionizing radiations are used. Non-ionizing rays have low energy with poor penetrative power while ionizing rays have high-energy and good penetrative power. Disadvantages of using UV rays include: low penetrative power, limited life

of the UV bulb, some bacteria have DNA repair enzymes that can overcome damage caused by UV rays, organic matter and dust prevents its reach, rays are harmful to skin and eyes and it doesn't penetrate glass, paper or plastic.

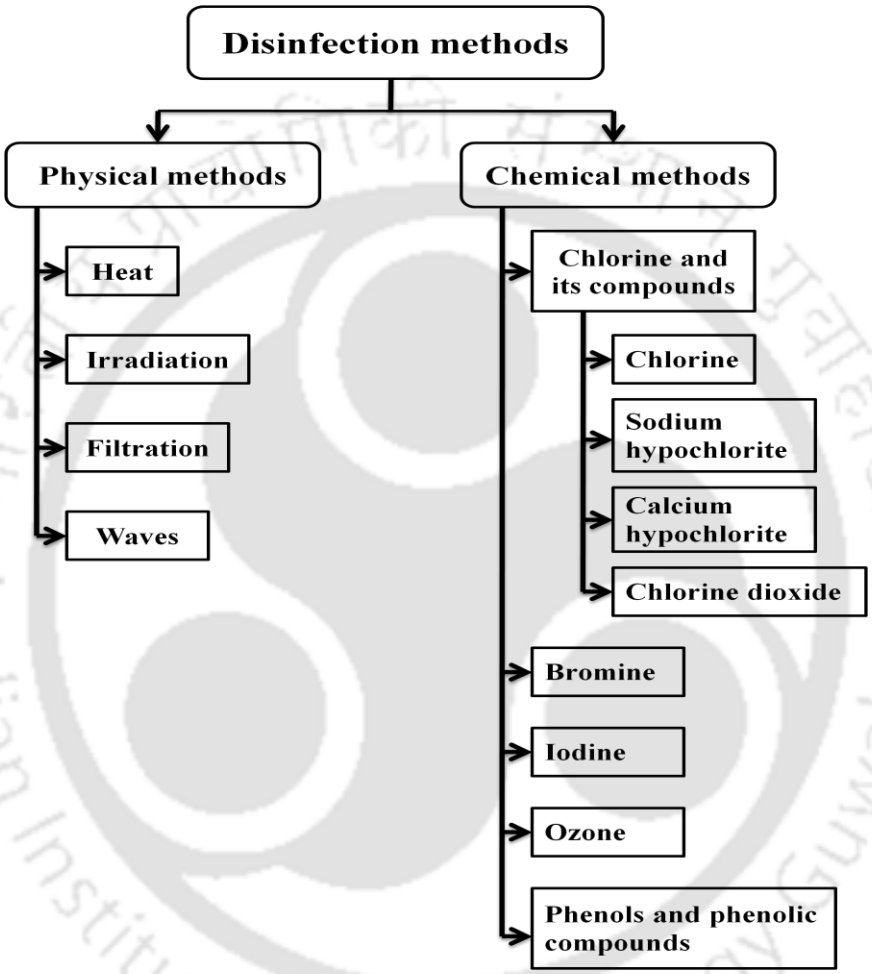


Fig. 2.3 Different physical and chemical methods commonly used for disinfection

Jung et al. (2011) studied the inactivation efficiency of several indigenous marine species, the formation of oxidants and other by-products using medium pressure ultraviolet (MPUV) ballast water treatment. Disinfection efficiency of the system was evaluated for surrogate microorganisms (*E. coli* and *Enterococci* group) and indigenous species of sea water (Busan >32

PSU) and brackish water (Nakdong river 20-22 PSU) at flow rates of 50 and 250 m^3/h. Results obtained were in accordance with the D-2 regulation of international marine organization (IMO). They also tested the water after MPUV for potential oxidants (H_2O_2, OH radicals and total residual oxidants) and halogenated by-products. Overall no harmful by-products were generated in the process.

Nourmoradi et al. (2012) studied the efficiency of UV irradiation for the inactivation of *Aspergillus sp.* employing continuous flow UV reactor. Inactivation efficiency was determined by the fungal density in water before and after irradiation by UV at fluence 4.15-25 mJ/cm^2. Results obtained showed 4 log reduction for *A. fumigatus, A. niger* and *A. flavus* using UV fluences of 12.45 mJ/cm^2, 16.6 mJ/cm^2 and 20.75 mJ/cm^2 respectively when their density was 1000 CFU/ml. They also measured the influence of turbidity and iron concentration on UV inactivation and found a decrease in inactivation efficiency with increase in turbidity and iron concentration.

Mc Kinney and Pruden (2012) studied the potential of UV to damage antibiotic resistant genes when present in both form either extracellular or within a host (antibiotic resistant bacteria ARB). Bacterial strains studied by them were methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococcus faecium* (VRE), *Escherichia coli* SMS- 3-5 and *Pseudomonas aeruginosa* 01. Results showed that Gram +ve ARBs (MRSA and VRE) were more resistant to UV disinfection than the Gram –ve ARBs (*E. coli* and *P. aeruginosa*). They also observed that higher doses of UV were required for ARGs (200 - 400 mJ/cm^2 for 3 - 4 log reduction) inactivation than ARB (10 - 20 mJ/cm^2 for 4 - 5 log reduction).

Guo et al. (2013) studied the disinfection of UV on both total heterotrophic bacteria and antibiotic resistant bacteria present in secondary effluent samples from a municipal wastewater treatment plant. Results showed effective inactivation of both total heterotrophic bacteria and all antibiotic resistant bacteria. The log reduction of nine types of antibiotic resistant bacteria varied between 1.0 and 2.4 after the UV treatment at a fluence of 5 mJ/cm^2. They also found microbial selectivity of UV disinfection for antibiotic resistant bacteria.

Bustos et al. (2014) studied the comparison between ozone (3-40 mg/l) and UV (8.5- 12 mJ/cm^2) for disinfecting primary effluent at pH 5 - 9. The result obtained showed about 72 – 78% inactivation of fecal and total coliform respectively by ozone at 20 mg/l dose and more than 80% inactivation for both using UV. Both the methods were found to be least effective at pH 7.

Beck et al. (2014) studied the infectivity of adenovirus at different UV wavelengths and compared them with DNA damage. They concluded that at wavelength 240 nm and below, the loss of infectivity was due to damage to a viral component but at wavelengths 253.7, 270, 280 and 290 nm the loss in infectivity occurred due to DNA damage.

Filtration: In this method microbes are not killed but separated out. The commonly used membrane filters to remove particles from solutions have pore sizes between 0.2-0.45 µm. They are used to remove microbes from heat labile liquids such as serum, antibiotic solutions, sugar solutions, urea solution. It could be aided by using either positive or negative pressure using vacuum pumps.

Studies have been performed on the role of membranes in water treatment and their application before disinfection by Mysore (2012). He observed the removal of microbial, particulate and

dissolved contaminants by combinations of microfiltration, ultrafiltration, nanofiltration and reverse osmosis which is a more effective disinfection downstream.

Seo et al. (2012) reported a high bacterial pathogen removal rate within a short contact time with Ag/Al $(OH)_3$ mesoporous nanocomposite film as compared to untreated Al foam filter. The Ag/Al $(OH)_3$ mesoporous nanocomposite film could be used as antimicrobial filters for tap water purification, wastewater treatment etc.

Waves: Sound waves of high frequency disrupt cells. Bacteria and some viruses get killed on exposure to the sound waves of frequency >20,000 cycle/second for one hour.

The application of ultrasound has been studied in several fields like medical (Kulier and Kapp, 2011), industrial, environmental engineering (Ince et al., 2001 and Mason et al., 2007) etc. On the other hand microwaves are not antimicrobial by themselves but the heat they generate causes microbial death.

Gao et al. (2014) studied the inactivation of bacteria and yeast at different growth phases under controlled temperature by high frequency ultrasound. Results revealed high frequency ultrasound to be highly efficient for inactivation of bacteria in both exponential and stationary phase and achieved more than 99% inactivation. The bacterial inactivation was found to take place due to the acoustic cavitation generated free radicals and H_2O_2. Yeast resistance to high frequency ultrasound was more than bacteria.

Kotecha et al. (2013) studied the disinfection of water contaminated with *Bacillus globigii* spores using atmospheric pressure microwave plasma (APMP). Most promising results were obtained when He- H_2 or O_2 were used as plasma gases. These result were attributed to the germicidal

ultraviolet radiation when He- H_2 plasma is used whereas to the metastable oxygen species in the oxygen plasma. They were able to achieve greater than $\log_5$ inactivation.

Antoniadis et al. (2007) studied disinfection of simulated and septic tank wastewaters by high intensity, low frequency ultrasound waves. Results showed complete elimination of *E. coli* within 20-30 minutes on irradiation at 24 kHz, 450 Wand the efficiency was found to decrease with decreasing intensity and frequency. They also observed continuous irradiation to be more effective then intermittent treatment. The irradiation of septic tank effluent prior to biological treatment resulted in 3-log reduction of total microbiological load. Bacterial cell elimination was irreversible as no reappearance of microorganisms occurred after 24 hours.

Studies conducted by Furuta et al. (2004) showed inactivation of *E. coli* XL1- Blue using high intensity ultrasonic waves from horn type sonicator. Inactivation of *E. coli* followed the pseudo first- order behaviour, with an increase in inactivation rate constant on increasing the vibration face amplitude. The formation of H_2O_2 was not observed below 3μm indicating that ultrasonic shock waves is more important for inactivation than indirect effect of OH radicals formed by ultrasonic cavitation. They found more than 99% of *E. coli* inactivation in 180 s sonication at amplitude of 3μm and 2 mm thickness of squeeze film.

Dehghani (2005) investigated the effectiveness of ultrasound waves on *E. coli* inactivation. Here the aqueous suspension of *E. coli* was exposed to the ultrasound waves of 42 kHz which showed some degree of inactivation. Inactivation by ultrasound waves occur mostly at high sonication time.

2.3.2 Chemical Disinfection

Chemical disinfectants which have been in use are (a) chlorine and its compounds (b) bromine (c) iodine (d) ozone (e) phenols and phenolic compounds etc. Chlorine is the most widely used

disinfectant throughout the world. The chlorine compounds commonly used for wastewater disinfection are chlorine, sodium hypochlorite, calcium hypochlorite and chlorine dioxide (Winward et al., 2008). Many countries have switched from chlorine to sodium hypochlorite due to safety concerns related to storage and transportation.

Chlorine: It is an effective disinfectant for non-turbid and non-alkaline water (i.e. pH is not above 8). If the water is turbid then the turbidity should be reduced either by natural filtration or by filtration before treatment. Chlorine persists in water as residual chlorine after dosing which minimizes re-contamination during storage and transport (Sadiq et al., 2004). While estimating the chlorine requirement for disinfection it should be considered that some amount of it will react with the substances present in water. Chlorine is available in various forms as calcium hypochlorite, sodium hypochlorite and chlorine gas.

Calcium hypochlorite (bleaching powder) is a powder with 30 to 70 percent of chlorine. It should be stored in a cool, dry location in a corrosion resistant container due to its high oxidising potential. Calcium hypochlorite is commonly used in solution for the disinfection of rural water supplies or in the form of tablets in household.

Sodium hypochlorite is a solution with about 1 to 18 percent chlorine. As it is mostly water so transportation is a problem so it is mostly used in home or for water supplies where transportation is not required.

Chlorine dioxide is more powerful oxidizing agent then chlorine and its disinfectant action is not as pH dependent as chlorine. Its residues are long lasting and controls the taste and odour. Chlorine dioxide does not react with ammonia upto significant extent and hence could be used

for disinfecting water with high ammonia content. It is an unstable compound so it should be generated on site by reacting sodium chlorite with an acid or chlorine.

Several studies have been conducted using chlorine, chloramine and chlorine dioxide as disinfectants. Taylor et al. (2000) studied the susceptibility of *Mycobacterium avium* against chlorine, chloramine, chlorine dioxide and ozone. Baker et al. (2001) reported the effect of disinfectants like chlorine, monochloramine and ozone on *Helicobacter pylori* and compared its susceptibility to that of *Escherichia coli*. John et al. (2005) studied the use of chlorine and ozone to disinfect *Encephalitozoon intestinalis* spores. Girones et al. (2014) studied the inactivation of hepatitis E virus and human adenovirus 2 in water using chlorine. They reported the inactivation kinetics of both the viruses to be equivalent and supported the use of chlorine disinfection as an effective strategy to control waterborne transmission of hepatitis E virus. Yang W. et al. (2012) studied the comparison between four types of chlorine disinfection i.e. single step disinfection, sequential disinfection, traditional sequential disinfection and mixed disinfectant disinfection. They reported sequential disinfection which showed synergistic effect to be most effective for *E. coli* inactivation because of the presence of low levels of chlorine dioxide. This study suggests that temperature has a positive effect on inactivation while alkaline conditions decreased inactivation. Findlay et al. (2012) found sodium bisulfite quenching system to successfully reduce sodium hypochlorite while maintaining the chlorate levels below target. Zhou et al. (2014) studied chlorine dioxide effect on cell integrity, toxin degradation and disinfection by product formation of *Microcystis aeruginosa*. Results reported that chlorine dioxide can inhibit photosynthetic capacity and also no integral cells were left at 1.0 mg/L dose of chlorine dioxide. The released intracellular organic matter contributed to the formation of disinfection by products like trihalomethanes and halo acetic acid. Murphy et al. (2014) reported 3- log inactivation of

Cryptosporidium Iowa oocysts using solution of 5 mg/L chlorine dioxide or solution containing 5 or 1.4 mg/L chlorine dioxide with addition of free chlorine. This method is seen as a promising alternative for hyperchlorination.

Iodine: Iodine is more stable than chlorine and is an effective disinfectant in non-turbid water. It is generally used to disinfect small volumes of water for personal use due to its high cost for disinfection at large scale.

Ozone: Ozone an unstable gas which is slightly soluble in water is an efficient disinfectant. It does not leave any residual in the water because of its instability, hence it is impossible to over dose with ozone. Due to ozonation bleaching of colour and removal of tastes and odour takes place.

Studies have been conducted on pilot scale by Drury et al. (2012) for full-scale tertiary system using membrane filtration and later ozone disinfection. Effective removal of coliform bacteria and enteric viruses was found by membrane filtration.

Ozone-on-demand (OOD) generator and control technology introduced by Neibeuer et al. (2012) matched the ozone production to real time demand. This resulted in reduction of unit-operating cost and produced more finished water.

Talbot et al. (2012) evaluated the disinfection of resistant microorganisms by ozone using laboratory analysis and evaluation of efficiency of plants. Zheng et al. (2014) studied the effectiveness of ozonation on the removal of organic residues and pathogenic microorganisms from municipal secondary effluent. Ozonation removed 15.49% DOC, 36.36% UV_{254}, 73.61% chroma, 37.29% di-n-butylpthalate (DBP), 14.6% di-2-ethylexylpthalate (DEHP) and above 80%

of organic pollutants at a dose of 6mg/l. Removal of fecal coliforms by ozonation were close to 10^3 CFU/L and by chlorination 10 CFU/L. On combining ozonation and chlorination increase in fecal coliform removal occurred.

2.3.3 Disinfection by-products and other emerging contaminants of water

While pathogenic organisms from drinking water pose primary health risk to humans, an unintended health hazard are also caused by chemical disinfection by-products (DBPs). Disinfectants used for killing harmful microorganisms are powerful oxidants capable of oxidizing the organic matter and bromide present in the water source (rivers, lakes, and many ground waters) and DBPs are formed. The most common disinfectants currently in use are chlorine, ozone, chlorine dioxide, and chloramines and each of them produces their own set of chemical DBPs in treated drinking water (Richardson, 1998). Rook (1974) is the first to report on the identification of chloroform and other trihalomethanes as the DBPs in chlorinated drinking water. If the high levels of bromide is present in the source water it poses great concern because it has been found to be a potent carcinogen in laboratory animals (Kurokawa et al, 1986). The previous health researchers have tried to understand the effects of chronic exposure to DBPs and focused on cancer or mutagenicity. New concerns are being raised about potential adverse reproductive and developmental effects, such as low birth weight, intrauterine growth retardation, and spontaneous abortion (Richardson et al, 2002). Now the DBPs from oxidation of bromide are being recognized as toxic. Many brominated DBPs have been shown to be more carcinogenic than their chlorinated analogs. Some preliminary studies have indicated that iodinated compounds could be more toxic than their brominated analogs (Plewa et al, 2003; Hunter III and Tugman, 1995). Studies have been performed to identify and quantify the DBPs

generated and more studies are being done to see the effect of DBPs and to reduce them. Neale et al. (2012) studied formation of DBPs by using in-vitro bio-analytical tools. Badawy et al. (2012) found preozonation/ enhanced coagulation/ activated carbon filtration treatment to be most effective for reducing DBPs. Dobrovic et al. (2012) reported decrease in the level of THMs up to 59% and influenced bromide ions role in ozonation by the use of silver nano particles. Stayner et al. (2014) found that increase in concentrations of brominated THMs are related to increase in the frequency of maternal binucleated lymphocytes, which acts as marker for genomic damage and is able to predict cancer risk in adults.

Many studies till now have reported about the generation of DBPs in water disinfected by traditionally used chemical disinfectants and also their potent effect on humans. Hence, there is a need to look for new technologies for disinfection. Among new technologies photosensitization looks very promising because it makes use of three non-toxic components: photoactive compound (photosensitizer), light and oxygen.

2.4 Photosensitization

Photosensitization is a treatment involving a photoactive compound (photosensitizer), light and molecular oxygen to achieve destruction of a biological target. In 1903-1905 the group of Von Tappeiner made very first attempts to apply photodynamic therapy for treatment of tumours and other skin diseases. The era of photosensitization was initiated by Oscar Raab a member of von Tappeiner group in 1900 as stated by Luksiene and Dougherty (Thandu et al., 2015). Raab observed death of *Paramecium caudatum* in presence of acridine orange on exposure to light. Bacteria and viruses when stained with dyes became photosensitive and lost their viability came into light in 1930s and later. In 1998 Wainwright acknowledged the dark activity associated with

photosensitizers as in case of cancer. Here the most important attribute is the selectivity of tumour cells over healthy tissues rather than the effects being purely mediated by illumination. Photodynamic technology has now been extensively developed for therapeutic purposes to selectively destroy cancer. This field also shows high potential for non-therapeutic purposes such as sterilisation including water sterilisation (Kuznetsova et al, 2007).

2.4.1 Types of Photosensitizers

The first FDA approved sensitizer is Photofrin which belongs to the porphyrin family and is derived from hematoporphyrin. Hematoporphyrin was produced by Scherer in 1841 by removing iron from blood and then treated with water (Moan and Peng, 2003). Several group of molecules which absorb in the UV-vis range have shown the ability to generate singlet oxygen. A photosensitizer should exhibit the following characteristics: Available in pure form of known chemical composition, synthesizable from available precursors and easily reproduced, High absorption coefficient in the spectral region of the excitation light A triplet state of appropriate energy to allow for efficient energy transfer to ground state oxygen, High quantum yield of the triplet state and long triplet sate lifetimes, High photostability. Fig 2.4 depicts different types of photosensitizers used for disinfection of water and wastewater along with microorganisms against which it is reported in literature.

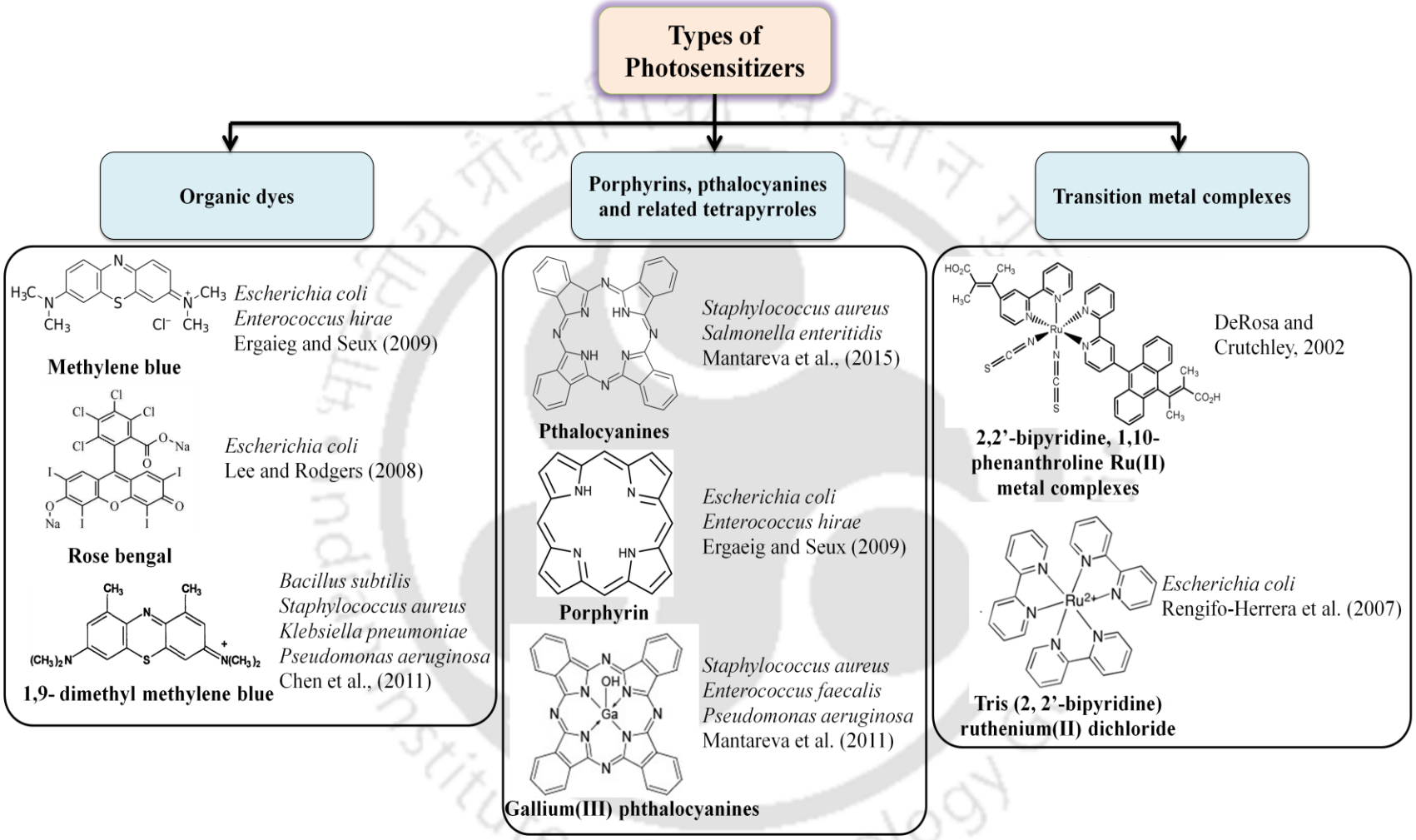


Fig. 2.4 Different types of photosensitizers used for disinfection of water and wastewater

Based on the chemical composition of dyes they can be classified as:

2.4.1.1 Organic dyes and aromatic hydrocarbons

Photosensitizers such as rose bengal, eosin and methylene blue are very effective because they have triplet state of appropriate energy to sensitize oxygen (Redmond and Gamblin, 1999). Methylene blue which belongs to phenothiazinium group has strong absorbance in the range 550-700 nm with a quantum yield of 0.52 whereas the absorption bands of xanthene dyes like rose bengal and eosin lies in the green area of the visible spectrum (480-550 nm) (De Rosa and Crutchley, 2002). When the number and atomic mass of halogen substituents are increased on xanthene skeleton, a shift towards red region is observed in the maximum peak. Aromatic hydrocarbons like naphthalene, anthracene and biphenyls have also been studied for their photosensitizer ability (Wilkinson and Abdel-Shafi, 1999). It was found through these studies that there is a competition between charge transfer interactions and energy transfer pathway. It was also observed that energy transfer pathway was of greater importance for biphenyls than for the napthalenes. If the nature of the substituents were changed on the biphenyl ring, the oxidation potential (EO_x) was affected which in turn changed their free energy of charge transfer, $\Delta G_{CT.}$ In these photosensitizers the higher oxidation potential lead to quenching by energy transfer pathway (DeRosa and Crutchley, 2002).

Several studies have been conducted employing organic photosensitizers for the disinfection of microorganisms in water. Jemli et al. (2002) studied the inactivation of fecal coliforms of wastewater using a combination of photosensitizers (methylene blue, rose bengal and cationic porphyrin) with sunlight on a small scale. In their study they found that the meso-substituted cationic porphyrin is more efficient and photostable as compared to methylene blue and rose

bengal in wastewater. Lower concentration (1μM) of cationic porphyrin caused little cell death whereas when concentrations were increased to 5 or 10 μM an increase in cell death was observed. They also concluded that log reduction in bacteria could also be increased by increasing the duration of irradiation and this could compensate for the low concentration of photosensitizer or for a less efficient photosensitizer.

Lee and Rodgers (2008) studied the laser flash photolysis for the production of triplet state of the xanthene dye and rose bengal and their reaction with oxygen to form singlet oxygen and superoxide anion radical. They also measured the yields of singlet oxygen and superoxide anion radical. Quenching of rose bengal by oxygen yielded approximately 75% singlet oxygen and 20% superoxide anion. They determined the reactivity of these species with nucleotides and DNA where only guanine residues showed any noticeable change at neutral pH.

Ergaieg and Seux (2009) used *Escherichia coli* (ATCC 25922) and *Enterococcus hirae* (ATCC 10541) as test strains for studying the photodynamic action of methylene blue and other compounds such as meso-tetra (4-N-methyl-4-pyridyl) porphyrin tetratosylate and rose bengal disodium salt. The concentration range 0.73 μmol/l to 3.65 μmol/l was tested for these photosensitizers. The results showed decrease in bacterial count as the concentration of photosensitizers was increased. Among all the tested photosensitizers meso-tetra (4-N-methyl-4-pyridyl) porphyrin tetratosylate showed maximum inactivation for both the strains whereas rose bengal was more effective in case of *Enterococcus hirae* and methylene blue in case of *E. coli*. This difference in behaviour of the organism was explained on the basis of charge and the structure of bacterial cell wall. Jori and Brown (2004) suggested that the positive charge favours the binding of photosensitizer molecule at critical cellular sites that once damaged by exposure to

light cause the loss of cell viability. Hence, positively charged photosensitizers are generally more efficient and can act at lower concentrations than neutral and anionic photosensitizer molecules.

Chen et al., (2011) studied the effect of pH on transient states of methylene blue and 1,9-dimethyl methylene blue using Gram positive (*Bacillus subtilis, Staphylococcus aureus and coagulase negative staphylococci*) and Gram negative (*Acinetobacter baumannii, Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens and Stenotrophomonas maltophilia*) bacterial strains. They suggested that spectroscopic data and protonation constant of triplet methylene blue ($^3MB^+$) (pKa= 7.2) at basic pH solution favours type II mechanism, while at low pH, the protonation rate of the central ring nitrogen is higher than the reaction with oxygen and the type I mechanism dominates resulting in radicals. At low pH, the triplet methylene blue ($^3MB^+$) is expected to be in the protonated $^3MBH^{2+}$ form before it reacts with oxygen to generate singlet oxygen. It was also revealed that weakly buffered solutions generate more singlet oxygen than strongly buffered solutions at the same pH due to the rate and degree of $^3MB^+$ protonation at each of these solutions. It was shown that bacterial photo inactivation rate increases for MB and DMB buffered solutions in the range of pH 5 to 9 which was justified as influence of pH on oxygen reactivity with MB and singlet oxygen generation. MB is thought to bind strongly to the walls of bacteria (Junqueria et al., 2002; Severino et al., 2003). MB may induce the form of both singlet oxygen and radical species especially at basic pH, which is expected to react with double bonds and lipids of bacteria walls and due to its hydrophilic and lipophilic character. From the obtained data they concluded that the inactivation time decreases by almost a factor of 25 between pH 5 and 9 for some bacteria while in others it was only by a factor of 3.

Kagel et al. (2015) studied the ability of gold nanoparticles (AuNP) to enhance the ability of photosensitizer rose bengal (RB) for antimicrobial activity. The experiments were carried out on Escherichia coli suspension either with RB or RB and AuNP. They studied relative concentrations of bacteria, photosensitizer and AuNP and also determined the survival rates of bacteria before and after of LED light illumination. Phototoxicity was checked for RB with and without AuNP for 10 and 20 minutes illumination. The results obtained showed decreases in the survival of bacteria with the increase in RB concentration and illumination time. They also reported an increase in lethal photosensitization of RB in presence of AuNP and proposed that this effect is due to plasmonic light enhancement, as the absorption spectra of RB and AuNP superimpose and favours electric field enhancement effects in the presence of AuNP. They also tested methylene blue for phototoxicity but did not found any difference in the presence of AuNP.

2.4.1.2 Porphyrins, pthalocyanines and related tetrapyrroles

The presence of porphyrins and their analogues in natural systems makes them ideal candidates for use in biological singlet oxygen generation. These photosensitizers generally lack cytotoxicity in the absence of light, which is important in some applications like photodynamic therapy of cancer. The porphyrins and their derivatives have the ability to absorb several wavelengths in the UV- vis range among which the major bands are Soret band in the blue and the Q-band in the red, which represent important components of sunlight. Many porphyrins have high quantum yields for long lived triplet states. The properties of porphyrins depend on the substituents of the macrocycle, metal ions coordinated at its centre, and ligands attached to the axial positions of the metal ion. Some porphyrins undergo rapid decomposition in the presence

of singlet oxygen (photobleaching). This could be deleterious in some industrial applications whereas advantageous in biological systems where rapid breakdown of the photosensitizer after use is necessary. Most extensively studied porphyrin for the production of singlet oxygen is hematoporphyrin with singlet oxygen quantum yield of 0.65 (Whorle et al., 1998).

Pthalocyanines, a class of intensely coloured macrocycles are the derivates of porphyrins (Leznoff and Lever, 1996). They differ from porphyrins by having nitrogen atoms to link individual pyrrole units. The peripheral benzene rings provide extended conjugation which helps in absorption at longer wavelengths. Light of longer wavelengths gives maximum penetration in the tissues which overlaps the absorption spectra of pthalocyanines making them ideal candidates for photodynamic therapy (PDT). Mantareva et al., (2015) studied photoinactivation of two pathogenic bacterial strains (methicillin-resistant *Staphylococcus aureus* - MRSA and *Salmonella enteritidis*) using phthalocyanine with irradiation. A greenish colored nanoparticles of phthalocyanine were synthesized by immobilizing dodecylpyridyloxy Zn(II)-phthalocyanine on a photocatalyst TiO_2 anatase. The author reports complete photoinactivation of gram-negative *S. Enteritidis* in this study.

These classes of photosensitizers have been studied for photodynamic therapy for long and now they have also found their way for disinfection of water. Jemli et al. (2002) and Ergaeig and Seux (2009) have studied the phototoxicity of photosensitizers methylene blue, rose bengal and cationic porphyrin for the inactivation of fecal coliforms and *Escherichia coli* and *Enterococcus hirae* respectively.

Alouini and Jemli (2001) have reported the use of porphyrin [tetra-(4-N-methyl-pyridyl) porphyrin tetra-tosylate T_4MP_yP)] for disinfection of wastewater carrying human helminth eggs.

On absorption of light by porphyrin a short lived toxic species is generated which is responsible for destruction of eggs. The microscopic observation of eggs done by them showed many types of ultrastructural alterations on exposure to T_4MP_yP and adequate intensity of light. They also observed increase in egg alteration with increase in T_4MP_yP concentration and irradiation time.

Lambrechts et al. (2004), they aimed at investigating the effect of cations Na^+ and Ca^{2+} on the photoinactivation efficiency of Gram –ve *Pseudomonas aeruginosa* and Gram +ve *Staphylococcus aureus.* They suspended the bacteria in the buffer containing meso-tetra(N-methyl-4-pyridyl)-porphyrin or meso-mono-phenyl-tri(N-methyl-4-pyridyl)-porphyrin as photosensitizers and various concentrations of Na^+ and Ca^{2+} and exposed the suspension to a light of 9 J/cm^2. Both the bacteria were equally sensitive to photoinactivation in the buffer without cations but showed a strong decrease in photoinactivation of both bacteria in presence of cations. *Pseudomonas aeruginosa* showed more decrease as compared to *Staphylococcus aureus* and Ca^{2+} being more effective than Na^+.

Carvalho et al. (2007) studied two sets of neutral and cationic porphyrins as photosensitizers for the inactivation of sewage bacteria. Cationic porphyrins showed high activity against Gram –ve bacteria inactivating 94-99.8 % of them at 5μM concentration and 270 minutes of irradiation by white light.

Costa et al. (2008) tested photoinactivation of a sewage bacteriophage by six cationic porphyrin derivatives with two to four charges. They exposed a phage suspension of 5×10^7 PFU/ mL to white light (40 W m^{-2}) for 270 min, at three different photosensitizer concentrations (0.5, 1.0 and 5.0lM). They found that tetra- and tricationic porphyrins were able to inactivate T4-like sewage phage to the limits of detection whereas dicationic porphyrins did not show any significant

decrease in phage viability. Complete inactivation of phage by tetra cationic porphyrin was observed at a concentration of 5.0 μM after 270 minutes. Hence, they concluded that the rate of bacteriophage photoinactivation and the efficiency of the photosensitizer vary with the charge and the substituents in the *meso*-positions of the porphyrin macrocycle.

Nitzan et al. (1992) studied the photosensitization efficiency of deuteroporphyrin (DP) in the presence of the polycationic agent polymyxin nonapeptide (PMNP) on *Escherichia coli* and *Pseudomonas aeruginosa*. It has been established previously that Gram-negative bacteria have complete resistance to the photodynamic effect of porphyrins. The present study showed that when the bacterial cultures are treated with a combination of DP and PMNP cell growth and viability was inhibited.

Caminos et al. (2008) studied the mechanistic of *Escherichia coli* inactivation by 5,10,15-tris[4-(3-*N*,*N*,*N*-trimethylammoniumpropoxy) phenyl]-20-(4-trifluoro methylphenyl) porphyrin iodide (A_3B^{3+}) when irradiated with visible light and compared its activity with that of 5,10,15,20-tetra(4-*N*,*N*,*N*-trimethylammonium phenyl) porphyrin *p*-tosylate ($TMAP^{4+}$). The results of electrophoresis showed damages on plasmid and genomic DNA. Structural changes with appearance of low density areas into the cells and irregularities in cell barriers, which could affect the normal cell membrane functionality were also revealed by transmission electron microscopy (TEM). Hence, they concluded that DNA photodamage could interfere with membrane function and be the cause of *E. coli* photoinactivation.

Ragas et al. (2010) showed that pathogenic microorganisms and viruses which are resistant to antibiotics can be successfully inactivated using photodynamic therapy. They used 5,10,15,20-tetrakis (N-methyl-4-pyridyl)-21H, 23H-porphine (TMPyP) and 5- mono (N-decyl-4-pyridyl)

10,15,20- tri (N-methyl-4-pyridyl)-21H,23H-porphine dihydrochloride tetrachloride (MDPyTMPyP) as photosensitizers to disinfect *E. coli* CECT101. The bacterial uptake of TMPyP was determined by fluorescence spectroscopy. Maximum uptake was observed at 8 µM bulk concentration of TMPyP resulting in 5 X 10^5 TMPyP molecules per *E. coli* cell whereas 16 µM concentration led to unacceptable dark toxicity assessed by colony formation assay. It is notable that the supernatant obtained after centrifugation showed no signal confirming that fluorescence is from the cell bound TMPyP molecules. It was concluded that a fraction of TMPyP molecules is bound to the external structure of the cell wall and the rest has been internalized because it is well known that TMPyP readily binds to nucleic acids, either intercalated between guanine-cytosine base pairs or groove bound at adenine-thymine sites as mentioned by Lang et al. (2004). The concomitant changes in lifetime of fluorescence from 1.7-2.5 ns shows intercalation as described by Paoli et al. (2002) and between 10-11 ns shows groove binding (Vergeldt et al., 1995). The values observed in this work are close to the values obtained earlier and hence they derived the conclusion that internalized TMPyP molecules are bound to the cytosolic nucleic acids. Subsequent, exposure to light induces formation of singlet oxygen both inside and outside the cell, thereby inactivating the bacteria.

Mantareva et al. (2011) synthesised and investigated the photodynamic activity of a cationic water soluble gallium(III) phthalocyanines (GaPcs) peripherally substituted with four and eight methylpyridyloxy groups, and absorbs visible light. They studied the cellular uptake and photodynamic activity for Gram +ve methicillin resistant *Staphylococcus aureus* (MRSA) and *Enterococcus faecalis* bacteria and Gram negative *Pseudomonas aeruginosa* bacteria and the fungus *Candida albicans* in planktonic phase. It was observed that cellular uptake of tetra-methylpyridyloxy substituted GaPc1 has lower uptake as compared to octa-methylpyridyloxy

substituted GaPc2. They compared the results of photodynamic activity of GaPcs with methylene blue (MB) and Zn (II) pthalocyanine with same substitution (ZnPcMe) and found high photoinactivation of microorganisms in planktonic phase as compared to the dark toxicity of GaPc1 towards MRSA and *E. faecalis* when treated with 3.0 μM GaPc1 at mild light conditions (50 J cm^{-2}, 60 mW cm^{-2}). At higher concentrations (6.0 μM) both GaPc2 and ZnPcMe showed complete inactivation of fungal biofilm grown on polymethylmethacrylate (PMMC) resin, whereas the bacterial biofilms were not susceptible to GaPcs with only 1-2 log reduction. For the inactivation of bacterial *E. faecalis* biofilm MB was found to be effective.

Spesia and Durantini (2013) studied the photoinactivation of *Streptococcus mitis* under different experimental conditions to understand about the photodynamic process and cellular uptake using zinc(II) 2,9,16,23-tetrakis[2-(N,N,N- trimethylamino) ethoxy] phthalocyanine ($ZnEPc^{4+}$). They observed a 3 log decrease in *S. mitis* survival in cell suspension when incubated with 2μM $ZnEPc^{4+}$ and irradiated for 30 minutes with visible light. Under continuous irradiation, broth with 5μM $ZnEPc^{4+}$ showed no growth. The studies on mechanism of photodynamic inactivation conducted by them showed protection of cells in presence of azide ion while mannitol did not show any effect. $ZnEPc^{4+}$ was found to strongly interact with calf thymus DNA but photoclevage of DNA occurs only at longer irradiation periods. They found mesosome like structural changes in *S. mitis* when observed under transmission electron microscope and 2 hour irradiation showed cytoplasmic segregation and photodynamic inactivation effects like variability in wall thickness of the cell wall. However, it was observed that photodamage to the cell envelop was insufficient to release intracellular biopolymers, so they concluded that may be the modifications in cytoplasmic biomolecules and alterations in the cell barriers are the cause of *S. mitis* photoinactivation.

Orlandi et al. (2013) synthesised two dicationic 5,15-di (N-alkyl-4-pyridyl) porphyrins, with methyl and benzyl N-alkylating groups and compared their antibacterial efficiency when irradiated with halogen-tungsten white lamp. They found that the antibacterial efficiency of the porphyrin carryoing benzyl group was higher than porphyrin with methyl group against pure and mixed cultures of microorganisms (*Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa* and *Staphylococcus aureus*). Benzylated photosensitizer showed better interaction with the bacterial strains as compared to methylated photosensitizer due to its lipophilic nature. The results obtained by them showed that better photosensitizer and cell binding is required for significant effect.

Alves et al. (2014) studied cationic porphyrin [5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin tri-iodide] coupled to cationized silica-coated magnetic nanoparticles of Fe_3O_4 (hybrid 6) which demonstrated antimicrobial activity for water disinfection through the photodynamic process. Later, they synthesised a new nanomagnet-porphyrin hybrid with $CoFe_2O_4$ core (hybrid 7) for inactivation of bacteria in water. Both the hybrids (6 and 7) showed photostability and retained their magnetic properties during photoinactivation experiments. The cumulative values of bacterial inactivation after a six-cycle reuse were approximately 42 $\log_{10}$ colony-forming units (CFU) mL^{-1} in 21.5 h with hybrid 6 and approximately 38 $\log_{10}$ CFU mL^{-1} in 27 h with hybrid 7.

2.4.1.3 Transition metal complexes

Most of the studies involve organic molecules for production of singlet oxygen in photosensitization but some inorganic complexes have been reported to be efficient photosensitizers. One of these complexes are of transition metal Ruthenium (II), which shows

strong absorption in the UV- vis regions of the spectrum. Many Ru (II) complexes have long lifetimes of emission from the triplet metal-to-ligand charge transfer states which makes oxygen quenching an efficient process in aerated solutions.

Many ruthenium-based photosensitizers have been found to be more efficient singlet oxygen producers than the organic photosensitizer like methylene blue, and are comparable to the widely used rose bengal (DeRosa and Crutchley, 2002). Demas et al. (1977) have studied the oxygen quenching of 16 luminescent diimine (2,2'-bipyridine, 1,10- phenanthroline and/or substituted phenanthroline) metal complexes of Ru(II), Os(II), and Ir (III). Recently, Garcia-Fresnadillo et al. (1996) examined the photosensitizer ability of a series of complexes $[RuL_3]^{2+}$ where L is 2,2'-bipyridine (bpy), 1,10-phenathroline (phen), etc.

Rengifo-Herrera et al. (2007) studied the inactivation of wild and collection type *Escherichia coli* by either TiO2 nanoparticles, water-soluble tris (2, 2'-bipyridine) ruthenium(II) dichloride or Rose bengal (RB) under sunlight irradiation and compared their efficiency. They investigated the concentration of dye, illumination time, photostability, presence of scavengers, and post-treatment regrowth of bacteria. Ru (II) complex showed better inactivation effect against *E. coli*, than TiO_2 and rose bengal after 1 hour of solar illumination due to combination of visible light absorption, photostability, and production of $^1O_2$ and other ROS when bound to the bacterial membrane. Both wild and collection type bacteria showed sensitivity to 2-10 mg/L of the dye. They concluded their study as the higher quantum yield of ROS generation by the sensitizing dyes compared to the semiconductor photocatalyst determines the faster sunlight activated water disinfection of photodynamic processes.

2.4.2 Light Sources for Photosensitization

Generally every visible light source with suitable spectrum and power can be used for photosensitization. In earlier days photosensitization was performed using conventional gas discharge lamps (Stables and Ash, 1995). To inactivate bacteria both light sources coherent (lasers) and non-coherent (lamps and light emitting diodes, LEDs) could be used. Lasers as coherent light sources were considered superior to the conventional light sources like incandescent lamps (Luksiene 2005). Fig 2.5 depicts different light sources that can be used for photoinactivation of microorganisms in water and wastewater.

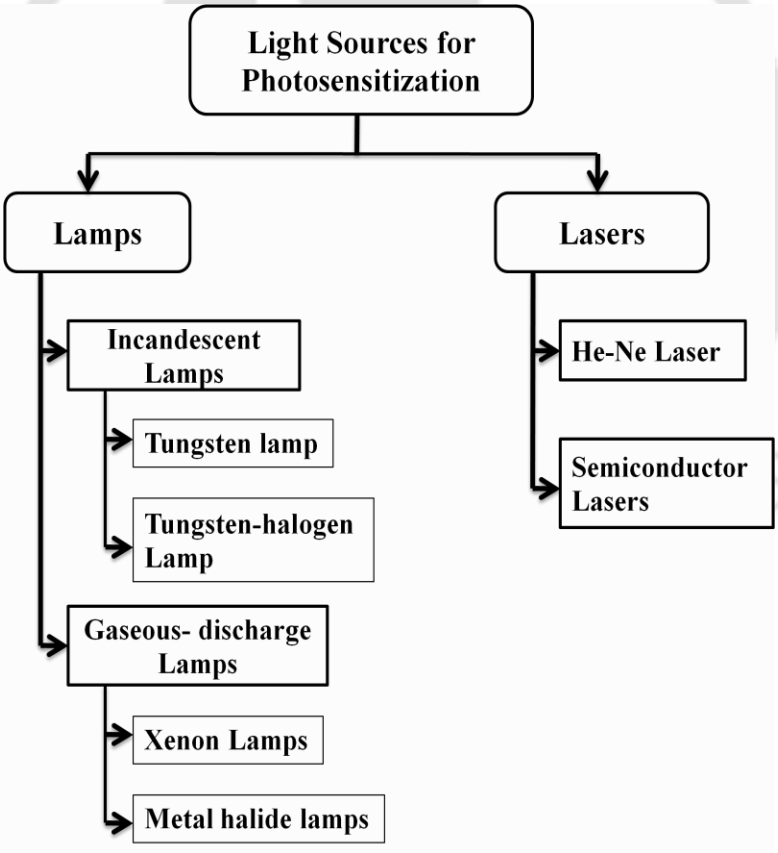


Fig. 2.5 Different light sources used for photoinactivation of microorganisms

2.4.2.1 Lamps

For the inactivation of bacteria two types of lamps are used one with continuous spectrum (incandescent lamp, xenon arc lamp etc.) and the other with spectrum in bands (gas discharge lamp or metallic vapour lamps).

Incandescent Lamps

A glass bulb with a tungsten filament and filled with an inert gas (tungsten-halogen lamp) or having a vacuum (tungsten lamp) are known as incandescent lamps.

Tungsten lamp:

These lamps function on the phenomenon of thermal emission. The filament is heated to incandescence by passing electric current and light is produced. Range of the temperature depends on the type of the filament (band, spiral or double spiral) and intensity of the current passed. The emitted light from these lamps should be filtered to narrow the spectrum band in the absorption maximum of the photosensitizer. It has been used to inactivate bacteria such as *Escherichia coli (Caminos et al., 2006), Staphylococcus aureus, Staphylococcus epidermidis (Tegos and Hamblin, 2006), Vibrio vulnificus (Wah et al., 2005)* etc. using different photosensitizers. Studies have also compared photoinactivation of Gram +ve and Gram –Ve bacteria with better results for Gram +ve.

Tungsten-halogen Lamp:

They are made of a wolfram filament in a quartz balloon filled with halogen gas. Its functioning is based on the regenerative cycle of the halogen gas. When these lamps are used the photosensitizer shows certain specificity to the pathogens (Banfi et al., 2006) even if they

are from the same class. Some studies also showed that photodynamic inactivation is dependent on radiation dose (Lambrechts et al., 2005 and Golding et al, 1998).

Gaseous- discharge Lamps

These lamps are based on the electrical discharge between two electrodes in a vapour or gas atmosphere and the radiation emitted depends on the gas in which discharge takes place (Calin and Parasca, 2008). The most common lamps among this category are mercury vapour lamps, hydrogen or deuterium lamps or xenon lamp. Only xenon lamp and metal halide lamp has been used to study photodynamic inactivation.

Xenon Lamps:

These lamps work at high pressure and temperature (around 900°C) giving high luminosity. Their emission spectrum is continuous in the ultraviolet-visible-infrared (UV-VIS-IR) domain. While using these lamps filters should be used to narrow the emission in the visible range because most of the photosensitizers have their absorption maxima in this range and also UV-B is well known to cause destruction of DNA in bacterial cells. Studies have confirmed dose related effect for both the photosensitizer and the radiation (Schafer et al., 2000 and Matevski et al., 2003).

Metal halide lamps:

Metal halide lamps are superior to mercury vapour lamp due to their light efficacy and better colour definition. Here, the discharge tube contains inert gases, a certain amount of mercury and a mixture of metal halides. Ashkenazi et al. (2003) found a comparable effect on Gram +ve and Gram –ve bacteria photoinactivation by using these lamps.

2.4.2.2 Lasers

Due to its monochromaticity and coherence it is considered as an ideal light source for photodynamic inactivation. Monochromaticity allows only the radiation of wavelength at which the photosensitizer has its absorption maxima and coherence offers the possibility to deliver light through optic fibres at the contaminated areas of the body.

He-Ne Laser:

This laser is built in a Pyrex glass tube filled with a mixture of helium and neon. There are two electrodes in the tube a nickel anode and an aluminium cathode which pass through the glass on wolfram rods. Two mirrors are axially aligned at the ends of the tube which acts as optical resonators of the laser. The spectral bands could be changed by using resonant cavities selectively.

He-Ne lasers have been intensively used for the photodynamic inactivation of bacteria specially *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Embleton et al, 2002; De Simone et al, 1999; Embleton et al, 2005 and Komerik et al, 2003).

Semiconductor Lasers:

It is a device based on the phenomenon that takes place at the junction of an extrinsic type n semiconductor and an extrinsic type p semiconductor. The semiconductor lasers (InGaAlP, GaAlAs, etc.) have been used for photodynamic inactivation of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Infected Plaque etc. (Griffiths et al, 1997; Carvalho et al, 2006 and Wilson and Patterson, 1986) with encouraging results.

2.4.3 Role of Oxygen

As mentioned earlier, photosensitization requires interaction between three non-toxic components: photosensitizer, light and oxygen. Photosensitization starts with the absorption of light photon by a sensitizer which results in the excitation of the molecules from the ground state (S_0) to the extremely unstable excited singlet state (S_1) with a half-life in the range of 10^{-6} – 10^{-9} s. This singlet state photosensitizer could now follow any of the three pathways i.e. it either decays back to the ground state by fluorescence or undergoes internal conversion where the energy is lost as heat to the surroundings. The third mode is to have an intersystem crossover to the longer lived (10^{-3}) triplet excited state (T_1). Cell destruction is most efficient when compounds with longer half-life and high quantum yield for the triplet excited state are used. Relaxation from the triplet excited state could result in phosphorescence or induction of two types of photo-oxidative reactions (Fig 2.6).

Type I mechanism involves hydrogen atom abstraction or electron transfer between the excited sensitizer and a substrate yielding free radicals. These radicals then react with the oxygen to form peroxides, superoxide ions and hydroxyl radicals which further initiate radical chain reactions. Type II mechanism is initiated by an energy transfer process with the ground state oxygen (Bissonette and Lui, 1997). Both the reactions occur simultaneously and are in competition with each other (Redmond and Gamlin, 1999). Hence, a series of cytotoxic reactions are triggered in the cells and the injuries induced lead to cell damage and death (Luksiene et al., 2003). The cell injuries induced by photosensitization include disruption of cell membrane, inactivation of different enzymes and damage of DNA (Demidova and Hamblin, 2004).

Dahl et al. (1987, 1989) noted that presence of bacterial cell wall lipopolysaccharide coat in Gram negative bacteria present a physical or chemical barrier which singlet oxygen produced outside of cells must pass to interact with a vital target such as membrane or cytoplasmic components. As a result some strains that fail to produce a large portion of the lipopolysaccharide have displayed greater sensitivity to exogenous singlet oxygen than do strains that retain this ability. Pure singlet oxygen mediated killing of Gram negative and Gram positive bacteria was compared. The bacterial strains used were *S. typhimurium, E. coli, Sarcina lutea* and *Staphylococcus aureus* and exposed to pure exogenous singlet oxygen on membrane filters. Results showed that *S. typhimurium* a mutant which lacks nearly all of the cell wall lipopolysaccharide coat manifests concomitant enhancement of penetration by some exogenous substance which responded to singlet oxygen with initially faster inactivation than did the *S. typhimurium* wild type strain , although maximum rates of killing appeared to be quite similar. They concluded that cell wall plays an important role in susceptibility to singlet oxygen. The outer membrane lipopolysaccharide portion of the Gram negative cell wall initially protects the bacteria from extracellular singlet oxygen, although it may also serve as a source for secondary reaction products which accentuate the rates of cell killing.

Valduga et al. (1993) studied a system known as separated surface- sensitizer. In this system the photosensitizer is separated from the substrate by a thin layer of air. Under such conditions only the singlet oxygen generated could react with the surface ruling out the possibility of Type I photosensitized process. They used this method to study singlet oxygen reaction with Gram +ve *Streptococcus faecium* and Gram –ve *Escherichia coli.* They noticed a drastic decrease in the survival of *S. faecium* as compared to *E. coli* when they were exposed to singlet oxygen for different periods of time. *E. coli* showed any sensitivity to the singlet oxygen only when the

outer membrane is altered by $CaCl_2$ or tris (hydroxymethyl) aminomethane-ethylenediaminetetraacetic acid (Tris-EDTA). Hence, from biochemical and ultrastructural analyses it is suggested that singlet oxygen damages the cytoplasmic membrane and the genetic material.

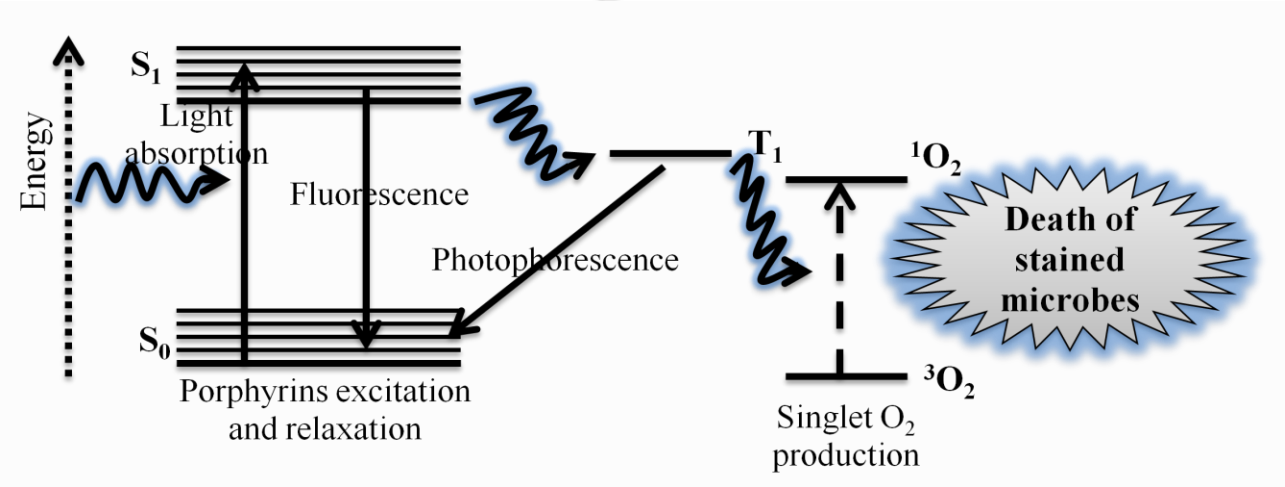


Fig. 2.6 Scheme of Photosensitization using porphyrin as model phtosensitizer (Luksiene and Zukaukas, 2009)

2.4.3.1 Properties of Singlet Oxygen

(a) Electronic structure of singlet oxygen

There are two low-lying singlet excited states for molecular oxygen, $^1\Delta g$ and $^1\Sigma^+ g$ (Fig 2.7). These states differ from each other only by the structure of the π-antibonding orbitals in the electronic configurations. As could be observed from above diagram the transition from the $^1\Delta g$ state to the $^3\Sigma^- g$ state is spin forbidden, thus the $^1\Delta g$ O_2 is a relatively long-lived species (De Rosa and Crutchley, 2002). The second excited state of oxygen, on the other hand, is short-lived due to a spin-allowed transition to the $^1\Delta g$ state. This difference in stability is confirmed by the

radiative lifetimes of O_2 ($^1\Delta g$) and O_2 ($^1\Sigma^+ g$); which are 45 min and 7- 12 s in the gas phase (Arnold et al, 1968) and 10^{-6}- 10^{-9} s, and 10^{-11} – 10^{-9} s in solution (Merkel et al, 1972).

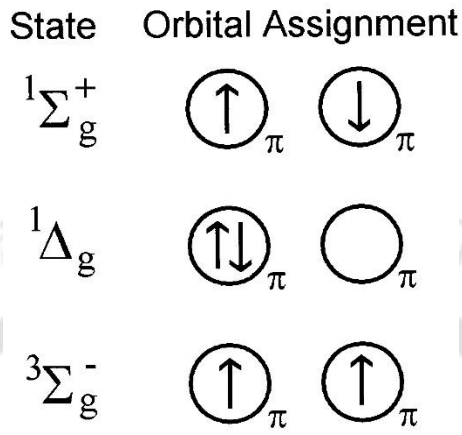


Fig. 2.7 Primitive representations of molecular oxygen lowest singlet and triplet states (De Rosa and Crutchley, 2002)

(b) Quenching of singlet oxygen

Once the molecular oxygen has reached its singlet excited state, to return to its ground state it should be deactivated which can take place as follows:

(1) Physical quenching: In this form of interaction the deactivation takes place without O_2 consumption or product formation.

(2) Chemical quenching: Here, the quencher reacts with the singlet oxygen to give new product (DeRosa and Crutchley, 2002).

2.4.4 Mechanism of Microbial Inactivation

The steps required for the photosensitization-based inactivation of a bacterial cell involves: (*i*) accumulation of the photosensitizer in the bacteria; (*ii*) translocation of the photosensitizer into the cytoplasm must be possible; (*iii*) there are two ways for explaining the lethal damage of bacteria: destruction of either DNA or membrane (Fig 2.8). After photosensitization with a wide

range of different photosensitizers both Gram +ve and Gram –ve bacteria have revealed breaks in both single and double-stranded DNA (Bertoloni et al, 2000 and fiel et al, 1981). *D. radiodurans*, is known to have a very efficient DNA repair mechanism but it is observed that it could be killed easily by photosensitization (Schafer et al, 1998 and Nitzan et al, 1999). The microorganisms could also be killed by damaging its cytoplasmic membrane which results in leakage of cellular contents. Alteration in proteins of cytoplasmic membrane was reported by Valduga et al. 1999.

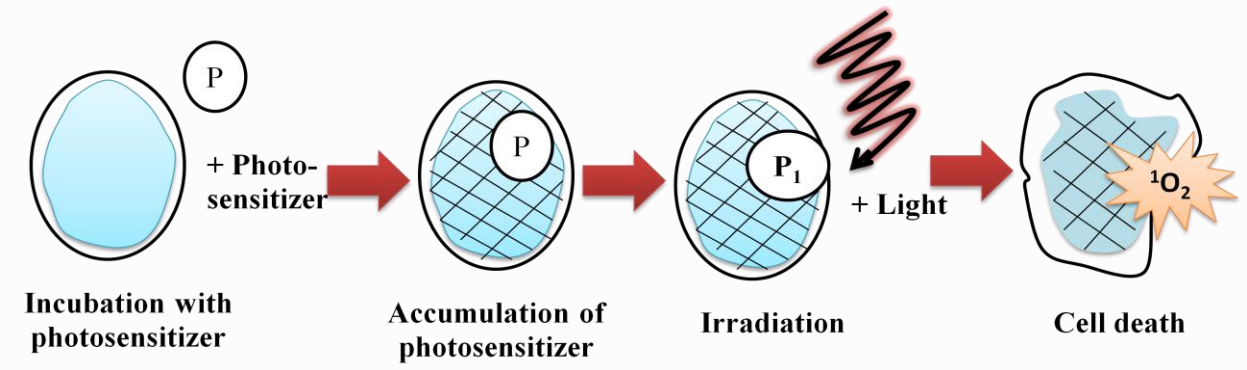


Fig. 2.8 Mechanism of destructive action of photosensitization in the cell: P- photosensitizer, P_1 – excited state of photosensitizer after absorption of light, $^3O_2$- triplet oxygen, $^1O_2$ – singlet reactive oxygen (Luksiene, 2005).

2.5 Design of Experiments

Design of experiments is a statistical design technique, which involves changing the levels of influential variables simultaneously, as compared to varying only one variable and its level at a time in conventional experimentation. The statistical interpretation of the results are in the form of analysis of variance (ANOVA), student's t-test, p-value, F-value etc. which give better understanding of the factor effects and their interaction on a given response. Statistical designs of experiments are necessary for systematic investigation requiring only a low number of experiments, and to interpret results in a meaningful manner (Montgomery, 2004). The factorial experimental design methodology involves changing all variables from one experiment to the

next. The reason for this is that variables can influence each other, and the ideal value for one of them can depend on the value of the others. This interaction between variables is a frequent phenomenon.

2.5.1 Factorial design of Experiments

The 2^k design is particularly useful in the early stages of experimental work, when there are many factors likely to be investigated. It provides the smallest number of runs with which 'k' factors can be studied in a complete design. Because there are only two levels for each factor, it is assumed that the response is approximately linear over the range of the factors level chosen, The statistical model for 2^k design would include k main effects, (k/2) two factor interactions, (k/3) three factor interaction and so on and one k-factor interaction. That means for 2^k design, the complete model would contain 2^{k-1} effects. The levels of the factors may arbitrarily be called "low" and "high" and denoted by "-" and "+" respectively. For example, in 2^2 design, two factor *viz.* A and B at two levels are chosen. The experimental design consists of treatment combinations denoted by (1) "a", "b", "c", "ab". A suitable number of runs carried out to replicate the levels of variables at their center point (0) provides an estimate of the residual error associated with the experiments and also the curvature of the response. Three degree of freedom is associated with the four treatment combination in 2^2 design, which in turn consists of two degrees of freedom associated with main effects of A and B, one degree of freedom associated with interaction effect between A and B (Montogomery, 2004).

2.5.2 Plackett Burman design of Experiment

Plackett-Burman designs are very efficient screening designs requiring minimal number of experimental runs than those required by a full- factorial design or classical one –factor at a time

approach (Araujo et al. 2016). They consider large main effects assuming that all interaction effects are negligible. These designs are useful for a rapid screening of the factors but has the risk of over evaluating some factors if the real effect lies on the interaction terms (Araujo and Brereton, 1996).

Plackett Burman design is constructed in the following manner: - the rows and the columns which represents the experimental combination of the parameters and the experimental parameters respectively. The first row (R_j) have almost the same number of positive and negative levels filled randomly and the rest of the design is constructed by shifting cyclically the columns $C_1 – C_i$ from R_j to $R_{j+1.}$ The cyclic permutation is carried out until the initial R_j is reached one more time. To avoid repeating the first experiment (R_j) the last row is filled with +1 or -1 symbols in a manner that every column contains equal number of +1 and -1 symbols (Araujo et al; 2016).

2.5.3 Analysis of Variance

Analysis of variance (ANOVA) is a collection of statistical models, and their associated procedures, in which the observed variance is partitioned into components due to different explanatory variables. In general terms, ANOVA explains any variation in the statistically derived model and significance of the model parameters. The model parameters, usually indicated in ANOVA, are the main effects, interaction effects and error terms, and their significance in the model is represented by Fischer ‘F’ and associated with P value. The other items in ANOVA table are degree of freedom (df), sum of squares (SS) and mean squares (MS). The MS value of a model term in an ANOVA table is obtained by dividing SS over df and its F value is obtained by dividing MS due to the model term by MS due to error. Normally, larger F

and lower P values of a model term in ANOVA indicate good significance of the term over others.

The fundamental technique is a partitioning of the total sum of squares into components related to the effects used in model. For example, we show the model for simplifies ANOVA with one type of treatment at different levels.

$$SS_{Total} = SS_{error} + SS_{Treatment}$$

The number of degrees of freedom (df) can be partitioned in a similar way and it specifies the chi-square distribution, which describes the associated sums of squares.

$$df_{Total} = df_{error} + df_{Treatment}$$

2.5.4 Student 't' Test

A t-test is any statistical hypothesis test in which the test statistic has a student's t distribution if the null hypothesis is true. It is applied when sample size are small enough that using an assumption of normality and the associated z-test leads to incorrect inference. The Student t-distribution in probability and statistics is a probability distribution that arise in the problem of estimating the mean of a normally distributed population when the sample size is small. It is the basis of the popular student t-test for the statistical significance of difference between two population means. The student t-distribution is a special case of the generalized hyperbolic distribution.

A test of the hypothesis is that the means of two normally distributed populations are equal. Given two data sets, each characterized by its mean, standard deviation and number of data points, one can use some kind of 't' test to determine whether the means are district, provided

that the underlying distributions can be assumed to be normal. All such tests are usually called Student t tests, though strictly speaking that name should only be used if the variances of the two populations are also assumed to be equal. There are different versions of the t test depending on whether the two samples are:

1. Independent of each other (e.g., Individual randomly assigned into two groups)
2. Paired, so that each member of one sample has a unique relationship with a particular number of the other sample (e.g., the same people measured before and after an intervention).

If the calculated P-value is below the threshold chosen for statistical significance (usually, the 0.05 level), then the null hypothesis, which usually states that the two groups do not differ, is rejected in favor of an alternative hypothesis, which typically states that the groups do differ.

1. A test of whether the mean of a normally distributed population has a value specified in a null hypothesis.
2. A test of whether the slope of a regression line differs significantly from 0.

Once a t value is determined, its corresponding P value can also be found using a table of values from Student's t-distribution.

It is clear from the preceding literature review that till date only a limited class of photosensitizing compounds have been examined for microbial inactivation. Moreover, no study has been conducted so far on the effect of individual process parameters affecting the microbial photo-inactivation process using such compounds. Hence, there is a need to study the parameters affecting the photo-inactivation process. There is also a need to study the effect of organic and

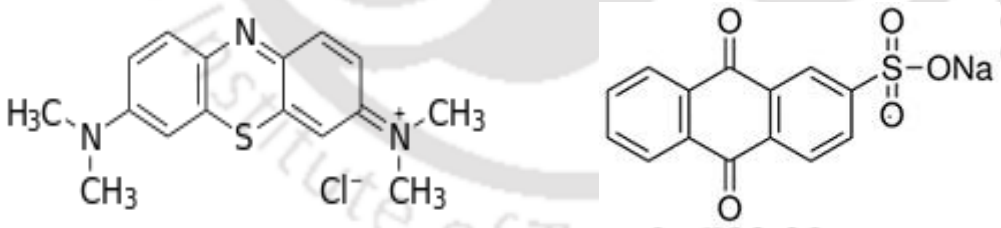
inorganic compounds commonly present in domestic wastewater on photoinactivation of microorganisms using photoactive dyes.

CHAPTER 3
MATERIALS AND METHODS

This chapter mainly deals with the methodology followed and the materials used in this research work. It essentially details the procedure for photo-inactivation of indicator microorganisms in aqueous solution under batch conditions employing photosensitive compounds.

3.1 Chemicals and reagents

Photo-sensitive compounds used in this study, methylene blue (MB) and sodium anthraquinone-2-sulphonate (SAQS), were purchased from Sigma Aldrich, Germany. Chemical structure of these cationic compounds is shown in Fig. 3.1. Phosphate buffer saline components (NaCl, KCl, Na_2HPO_4 and $KHPO_4$) and agar were purchased from Merck, India. Analytical reagents propidium iodide, dihydrochlorofluorescin diacetate (DCFDA), tri carboxylic acid (TCA), guanidine hydrochloride and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma Aldrich, Germany. Other chemicals, such as thiobarbituric acid (TBARS), 1,1,3,3-tetraethoxy propane etc., were obtained from Merck (India).



(a) (b)

Fig. 3.1 Chemical structure of the photosensitive compounds used in this study: (a) Methylene blue (M.W = 319.85) (b) Sodium anthraquinone-2- sulphonate (M.W= 310.26)

3.2 Microorganisms and its maintenance

All photo-inactivation studies using MB and SAQS were carried out using the well-known indicator organisms, viz, *Escherichia coli* and *Enterococcus hirae*. The bacterial strains were

procured from Institute of Microbial Technology (IMTECH), Chandigarh, India with the accession number MTCC 1610 and MTCC 3612, respectively. These strains were grown as per the provider's instruction, i.e. for 24 hours at 37°C and 180 rpm in Erlenmeyer flask containing nutrient broth for *E. coli* and brain heart infusion broth for *E. hirae*. The culture was maintained at 4°C in a refrigerator.

3.3 Seed culture preparation

Depending on the type of strain to be grown for the photo-inactivation experiments, 50 ml of Erlenmeyer flask containing 20 ml of either nutrient broth or brain heart infusion broth was autoclaved at 121°C for 20 minutes. The sterile media was then inoculated with the fresh grown bacteria of 1 ml inoculum volume. The flask was then incubated for 24 hours at 37°C and 180 rpm in a rotating orbital incubator shaker.

3.4 Photoinactivation of *E. coli* and *E. hirae* in aqueous solution using methylene blue

Inactivation experiments were carried out by transferring 1ml, 24 hour grown culture in 1.5 ml eppendorf tube and centrifuging the biomass at 10,000 × g for 10 minutes. The pellets obtained were washed twice with phosphate buffer saline (PBS) of respective pH. The washed pellets were resuspended in phosphate buffer saline (PBS) set at different initial pH 7.5, 8.25, 9.0 followed by serial dilution up to 1000 times. 10 µl of the suspension from 10, 100 and 1000 dilutions were spread on agar plates for initial viable colony counts. The PBS suspended cultures were then added with MB from its 1mM stock solution to ensure initial concentration in the range of 0.73µmol/l - 1.25µmol/l. Two set of mixtures were kept under dark condition on a gel rocker platform for three different incubation periods of 5, 15 and 30 minutes with constant shaking. After dark incubation one set was used as dark controls by spreading 10 µl of suspension on agar plates and the other set was exposed to a light intensity of 2700 lux for 10 minutes using 11W compact fluorescent light (CFL) in a closed chamber.

Photo sensitized bacterial suspensions (10µl) were then spreaded on brain heart infusion agar for *E. hirae* and nutritive agar plates for *E. coli*, set in duplicates. All the plates were incubated at 37°C for 24 hours. Viable cells in the culture plates were enumerated by colony counting method (Vilela et al. 2012), which involves counting of distinct viable colonies using a colony counter.

The average results of percentage inactivation of microorganisms from each duplicate runs in the study were calculated as per the following equation

$$\%\, inactivation = \left(C_i - \frac{C_f}{C_i}\right) * 100 \qquad (1)$$

Where, C_i and C_f are the initial and final viable cell counts.

3.4.1 Effect of concentration of photosensitizer, pH of solution and dilution

Photo-inactivation experiments were carried out to first study the combined effect of methylene blue concentration, cell suspension pH and initial viable cell count (dilution) employing the statistically valid full factorial design of experiment. The range and levels of these variables used in this study are presented in Table 3.1. Table 3.2 presents the experimental variables and their levels chosen in each of the experimental runs.

Table 3.1 Range and level of the variables used in the photo-inactivation experiment using Methylene blue as the photo-inactivating compound

Factors	**Low Level (-1)**	**Centre Point (0)**	**High Level (+1)**
Concentration of methylene blue	0.73 µmol/l	0.99 µmol/l	1.25 µmol/l
pH	7.50	8.25	9.00
Dilution	10	100	1000

Table 3.2 Combination of parameters and their levels used in the photo-inactivation experiments with Methylene Blue as the photo inactivating compound

Experimental run no.	Coded levels of the variables		
	MB initial concentration (µmol/L)	**pH**	**Dilution**
1	0.73	7.5	10
2	0.73	7.5	1000
3	0.73	9.0	10
4	0.73	9.0	1000
5	0.99	8.25	100
6	1.25	7.5	10
7	1.25	7.5	1000
8	1.25	9.0	10
9	1.25	9.0	1000

The initial concentration of photosensitizer was chosen as 0.73 µmol/l based on a report by Ergaieg and Seux, (2009) and Methylene Blue and water suspension was kept in an alkaline range to mimic natural water environment. For ensuring viable cell count, suspension containing the bacterial culture was diluted up to 1000 times as dilution beyond this value did not yield any viable colonies upon culture. For diluting the bacterial culture, 1 ml of freshly grown bacterial culture was centrifuged at 10, 000 ×g for 10 minutes. The pellet obtained is then washed and re-suspended in phosphate buffered saline (PBS). The serial dilution method was followed using Phosphate Buffer Saline (pH 7.2-7.4) up to 10^{-6} by transferring 1 ml of suspension from the previous test tube into the next, as shown in Fig. 3.2. The serially diluted suspension (10 µl) was then spread on agar plates and incubated at 37°C for 24 hours. Later, viable colonies growing on the agar plates were counted using a colony counter.

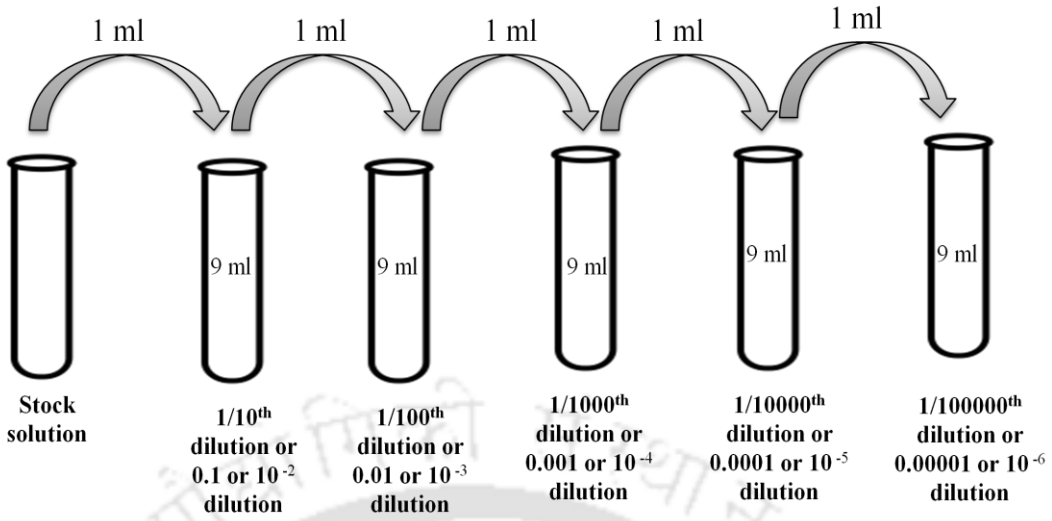
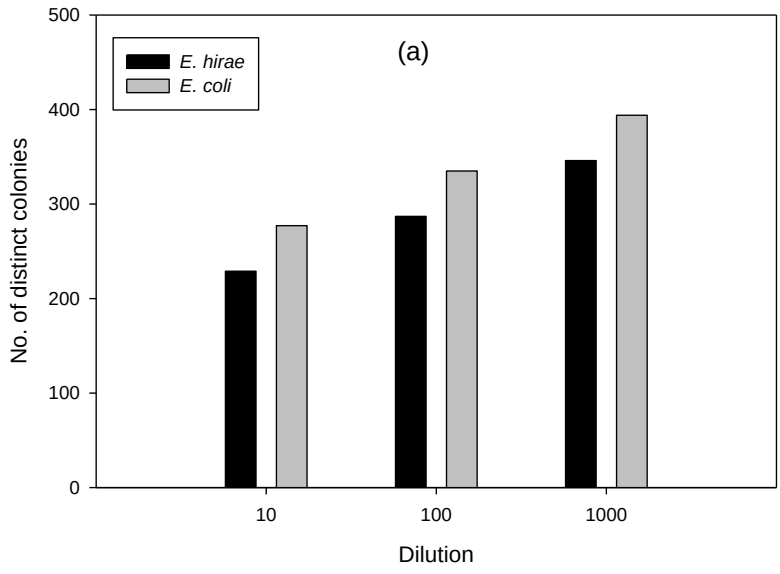


Fig. 3.2 Schematic representation of serial dilution method

3.4.2 Effect of dark incubation on photo inactivation efficiency

For photo-inactivation of micro-organisms, dark incubation with constant shaking is required to get a homogeneous mixture of bacterial suspension and the photosensitive dye (Ergaieg and Seux, 2009). Fig 3.3 shows initial colony count for *E. hirae* and *E. coli* with 10, 100 and 1000 dilution with both MB and SAQS.



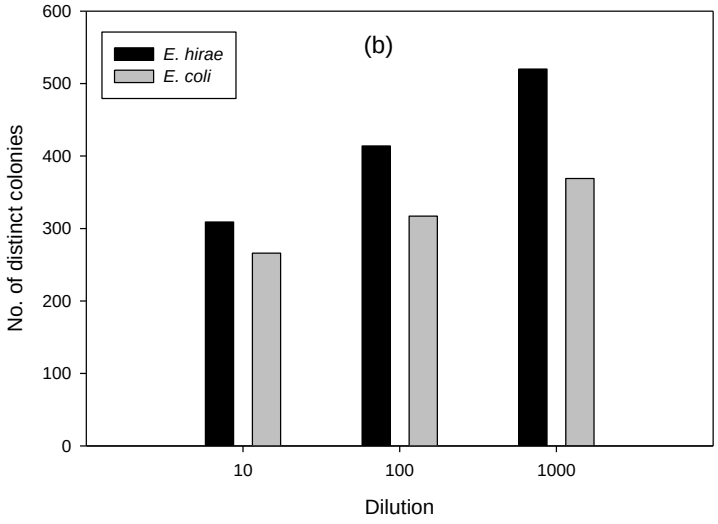


Fig. 3.3 Initial colony count with 10, 100 and 1000 dilution for *E. hirae* and *E. coli* (a) with MB (b) with SAQS

Hence in order to investigate the effect of dark incubation period on photo-inactivation using Methylene Blue, three different periods of dark incubation 5, 15, 30 minutes with constant shaking on a gel rocker has been investigated. For each set of experiments the Methylene Blue concentration was in the range 0.73 µmol/l to 1.25 µmol/l, dilution was upto thousand times and pH was in the range 7.5 to 9.00.

3.4.3 Studies on cell death using Flow cytometry

In order to confirm the results of microbial inactivation obtained from the photo inactivation experiments using the colony counting method flow cytometry method was used. Photosensitized bacterial cells from the previous experiments as explained in section 3.4 were obtained by centrifuging the cell suspension at 10000 × g for 10 minutes and the pellet was washed with PBS of a suitable pH. Later, the pellet was suspended in 1 ml of PBS followed by addition of 20 µl of propidium iodide (PI) solution (obtained by dissolving in distilled water in the ratio 1:1) and incubation in dark for 15 minutes. 1 ml sample was then analysed

for 100000 cells using BD FACS Calibur™, USA, flow cytometer equipped with an argon laser (L1) (wavelength: 488nm; Fluorescence channel: FL-2 yellow). PI fluorescence was measured to distinguish between the live and dead bacterial cells.

3.4.4 Mechanism of photo inactivation of bacteria

To gain further insight into the mechanism of photo inactivation analysis, reactive oxygen species (ROS), lipid peroxidation and protein carbonyl index were carried out.

3.4.4.1 Reactive Oxygen Species (ROS) determination

Sample from each photo inactivation experimental run as presented in Table 2 were added with 20µl of 20µM dihydrochlorofluorescin diacetate (DCFDA) and incubated for 30 minutes at 37°C. The suspension was then added with MB and kept in the dark on a gel rocker for 30 minutes. Later, it was exposed to visible light. Following the light exposure period of 10 mins, the suspension was spread on agar plates to check for viable cell count. The suspension was checked for 2, 7 dichlorofluorescin (DCF) fluorescence by excitation at 488nm and emission spectra was analysed in the range 510- 540 nm using Fluoromax 4.

3.4.4.2 Lipid peroxidation assay

Bacterial cell suspension was prepared and inactivation experiments were carried out as described in section 3.4. Experiments were carried out only for 10 time dilutions because higher dilutions didn't have enough precipitate. The treated bacterial cells were obtained in the form of pellet and lipid peroxidation products of cell lysate were determined as thiobarbituric acid reactive substances (Trivedi, 2005). Bacterial pellet was resuspended in PBS of respective pH (7.5 and 9.0) and sonicated by keeping on ice (4°C) using probe sonicator. An aliquot (100 µl) of bacterial lysate was allowed to react with 10%, trichloroacetic acid (200 µl) for 15 minutes on ice (4°C). Later, centrifuged at 3000 ×g for 15 minutes at 4°C to get the supernatant. The supernatant obtained is made to react with

thiobarbituric acid in ratio 1:1 by placing in boiling water bath for 10 minutes. The mixture is then cooled and absorbance is taken at 532 nm using Tecan plate reader to determine thiobarbituric acid reactive substances using 1, 1, 3, 3 - tetraethoxy propane as the standard. The equation obtained by fitting the linear trend line is used to calculate the value of lipid peroxidation in different experimental runs.

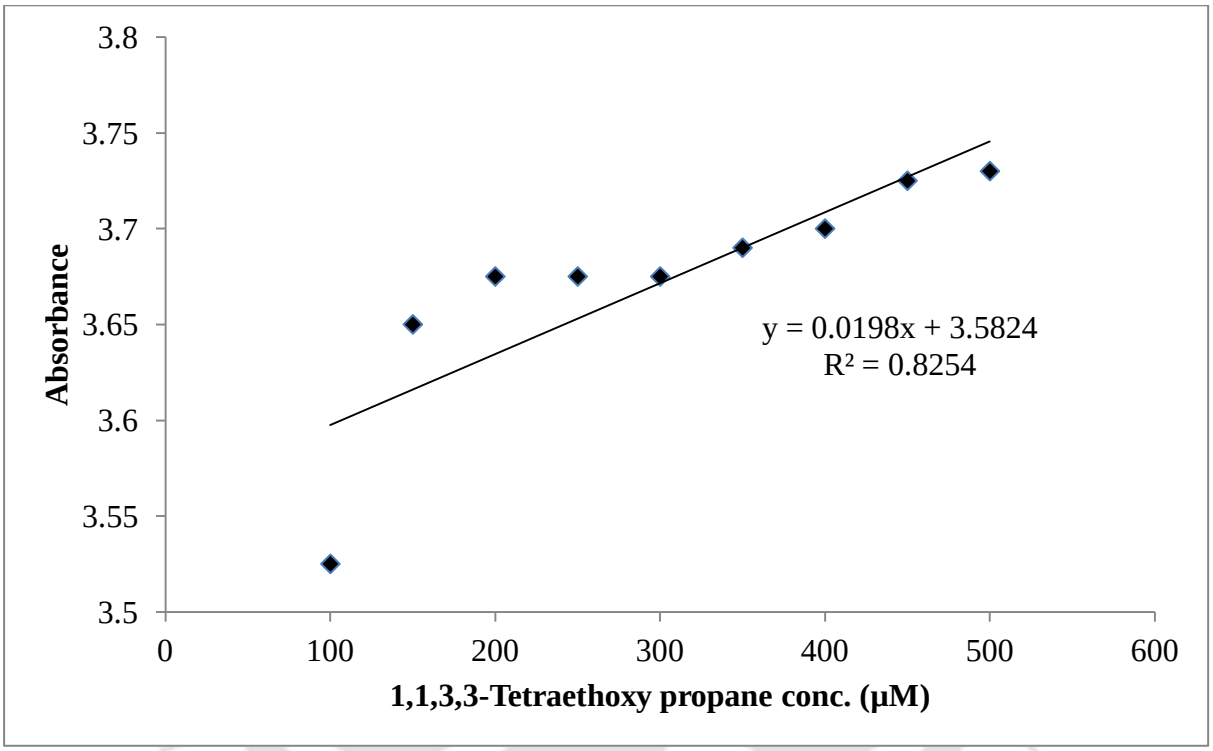


Fig. 3.4 Standard Curve for Lipid peroxidation assay

3.4.4.3 Estimation of Protein carbonyl

Higher dilutions didn't have enough precipitate so experiments were carried out only for 10 time dilutions. The pellets of treated bacterial cells were washed with PBS and resuspended in PBS of respective pH (7.5 and 9.0). Cell suspension was lysed by using probe sonicator by keeping the samples on ice (4°C). Lysate was divided equally in two equal portions and added with an equal volume of 10% trichloroacetic acid at 4°C. The mixture was incubated for 15 minutes at 4°C and then centrifuged at 3000 × g for 15 minutes. The precipitate obtained in one of the two portion was added with 500 µl of 0.2% 2,4-dinitrophenylhydrazine

(DNPH) in 2N HCl and the other portion was added with 500 µl of 2N HCl. The mixtures were then incubated at 37°C for one hour with continuous vortexing followed by addition of 55µl of 100% TCA for precipitating the protein. Samples were centrifuged and the pellet obtained was washed with a mixture of ethanol and ethylacetate. The pellets were then suspended in 600 µl of 6M guanidine hydrochloride followed by incubation for 30 minutes. Absorbance of the final mixture was recorded at 370 nm (Castegna et al., 2003) using Tecan elisa plate reader.

3.5 Photoinactivation of *E. coli* and *E. hirae* in aqueous solution using Sodium anthraquinone-2- sulphonate

The experimental design and the range of the parameters used were as defined in Table 3.3 and Table 3.4 respectively. The experiments were conducted by transferring 1ml of 24 hour grown bacterial culture in 1.5 ml eppendorf tube and centrifuging the biomass at 10,000 × g for 10 minutes. The pellet obtained is washed and used further for the experiment. The experiments were carried out as described under section 3.4. The light source used for these experiments was UV-A instead of white light.

Table 3.3 Range and level of the variables used in the photo-inactivation experiment using SAQS as the photo-inactivating compound

Factors	Low Level (-1)	Centre Point (0)	High Level (+1)
Concentration of SAQS (µmol/l)	0.73	0.99	1.25
pH	7.50	8.25	9.00
Dilution	10	100	1000

Table 3.4 Combination of parameters and their levels used in the photo-inactivation experiments with sodium anthraquinone-2- sulphonate as the photo inactivating compound

Experimental run no.	Coded levels of the variables		
	SAQS initial concentration (µmol/L)	**pH**	**Dilution**
1	0.73	7.5	10
2	0.73	7.5	1000
3	0.73	9.0	10
4	0.73	9.0	1000
5	0.99	8.25	100
6	1.25	7.5	10
7	1.25	7.5	1000
8	1.25	9.0	10
9	1.25	9.0	1000

3.5.1 Effect of Concentration of Photosensitizer, pH of Solution, Dilution and dark incubation

The process parameters and dark incubation period showed similar effects as described under the sections 3.4.1 and 3.4.2.

Discrimination between live and dead bacterial cells is also studied by propidium iodide (PI) fluorescence to simulate the results from colony counting method. The experiment was carried out as explained under section 3.4.3.

3.5.2 Mechanism of photo inactivation

To further understand how the bacterial inactivation is taking place, measurement of reactive oxygen species (ROS) have been done and later it is studied how these ROS help in

inactivation by measuring lipid peroxidation and protein carbonyl index. The experiments were carried out as explained under the sections 3.4.4.1, 3.4.4.2 and 3.4.4.3.

3.6 Inactivation of *E. coli* and *E. hirae* in aqueous solution using Ultraviolet light

Statistically valid full factorial design of experiment was used to assess the relationship between UV intensity ($\mu J/cm^2$) (energy), pH of the suspension and dilution on the inactivation of *E. coli* and *E. hirae*. The experimental design and the range of the parameters used were as defined in Table 5.

The experiments were conducted by transferring 1ml of 24 hour grown bacterial culture in 1.5 ml eppendorf tube and centrifuging the biomass at 10,000 × g for 10 minutes. The pellet obtained is washed and re-suspended in the respective pH buffers. These suspensions were then serially diluted upto 1000 times in PBS of pH 7.5, 8.25 or 9.0 according to the experimental design. 10 µl of the suspension from 10,100 and 1000 dilutions were spread on agar plates for initial viable colony counts. The PBS suspended cultures were then exposed to ultraviolet light of intensity varying from 50 $\mu J/cm^2$ to 100 $\mu J/cm^2$ using UV-C crosslinker. Duplicates of agar plate were then made by spreading 10 µl of bacterial suspension and incubated at 37°C for 24 hours in the incubator. Viable cells in the culture plates were enumerated by colony counting method (Vilela et al. 2012). Briefly, in this method the distinct viable colonies are counted using a colony counter.

The average results of percentage inactivation of microorganisms from each duplicate runs in the study were calculated using equation 1 under section 3.4.

Table 3.5 Range and level of the variables used in the photo-inactivation experiment using UV as the photo-inactivating compound

Factors	Low Level (-1)	Centre Point (0)	High Level (+1)
Energy of UV-C	50 µJ/cm^2	75 µJ/cm^2	100 µJ/cm^2
pH	7.50	8.25	9.00
Dilution	10	100	1000

3.7 Combined effect of MB and SAQS on photoinactivation of *E. coli* and *E. hirae*

From the previous experiments higher bacterial inactivation was obtained in experimental run with 1000 times dilution and 9.0 pH. These experiments were carried out with individual photosensitizer. Hence to understand the combined effect of both the photosensitizer on photoinactivation batch experiments were designed using statistical tool. The experiments were planned as per full factorial design (Table 6). The low and high levels of the PS are taken as 0.73 µmol/l and 1.25 µmol/l respectively from the previous experiments as explained under section 3.4 and the pH and dilution was taken as 9.0 and 1000 times respectively.

Table 3.6 Combination of photo sensitizers and their levels used in the photo-inactivation experiments

Experimental run no.	Coded levels of the variables	
	MB (µmol/l)	SAQS (µmol/l)
1	0.73	0.73
2	0.73	1.25
3	0.99	0.99
4	1.25	0.73
5	1.25	1.25

3.7.1 Photo-inactivation experiment with both MB and SAQS

Inactivation experiments were carried out by transferring 1ml, 24 hour grown culture in 1.5 ml eppendorf tube and centrifuging the biomass at 10,000 g for 10 minutes. The pellets obtained were washed twice with phosphate buffer saline (PBS) of pH 9.0 and re- suspended in it followed by serial dilution up to 1000 times. One set of PBS suspended 1000 times diluted bacterial suspension added with a combination of MB and SAQS from their respective 1mM stock solution as per the design (Table 3) were kept in dark as dark control. The other set added with the dyes were kept under dark condition on a gel rocker for 30 minutes with constant shaking and later exposed to a light of intensity 1500 lux (measured by a digital luxmeter) for 10 minutes using two 6 W tube light and two 6 W UV-A tube lights in a closed chamber. Photo sensitized bacterial suspension (10µl) was then spread on brain heart infusion agar plates and nutrient agar plates for *E. hirae* and *E. coli* respectively and incubated at 37°C for 24 hours. The experiments were carried out in triplicates and viable cells in the culture plates were enumerated by colony counting method as explained in section 3.4.

The percentage inactivation of microorganisms from each duplicate runs in the study was calculated using equation 1 under section 3.4.

3.7.2 Mechanism of photoinactivation

According to our hypothesis, the death of the microorganisms occur due to reactive oxygen species (ROS). So to confirm whether ROS is being produced during the reaction and the death is due to it we tried to measure ROS. For measuring ROS generated a fluorescent dye dihydrochlorofluorescin diacetate (DCFDA) was employed and the fluorescence was studied using Fluoromax 4 as detailed under section 3.4.4.1.

3.8 Photoinactivation of *E. coli* and *E. hirae* in synthetic wastewater using MB

The experiments were designed to study the effect of synthetic wastewater components on bacterial photo-inactivation employing Plackett Burman. Experiments were designed using concentrations of the components as 0 and +1.The components and their concentrations used are summarized in Table 7. All experiments were carried out by transferring 1 ml of 24 hour grown bacterial culture into 1.5 ml eppendorf tube and centrifuging the biomass at 10,000 × g for 10 minutes.

Table 3.7 Synthetic wastewater composition

Sl. No.	Components	Concentration (mg/l)
1	Urea	91.74
2	Ammonium chloride	12.75
3	Sodium acetate	79.37
4	Peptone	17.41
5	Potassium dihydrogen phosphate	23.40
6	Ferrous sulphate	5.80
7	Starch	122.0
8	Milk powder	116.19
9	Yeast	52.24
10	Copper chloride	0.536
11	Manganese sulphate	0.108

3.8.1 Photo-inactivation experiment

The pellets obtained were resuspended in phosphate buffer saline (PBS) set at pH 9.0 followed by serial dilution up to 1000 times. 10 µl of the suspension from 1000 dilutions were spread on agar plates for initial viable colony counts. The PBS suspended cultures were then added with combination of synthetic wastewater components (Table 7) according to the Plackett Burman design as described in Table 5. Later, MB from its 1mM stock solution was added to ensure initial concentration to be 1.25µmol/l. The mixtures were then kept under dark condition on a gel rocker platform for 30 minutes with constant shaking. After dark

incubation the suspension was exposed to a light intensity of 2700 lux for 10 minutes using 11W compact fluorescent light (CFL) in a closed chamber. Photo sensitized bacterial suspensions (10µl) were then spreaded on brain heart infusion agar and nutritive agar plates, set in duplicates. All the plates were incubated at 37°C for 24 hours. Viable cells in the culture plates were enumerated by colony counting method. The percentage inactivation of microorganisms from each duplicate runs in the study was calculated using equation 1 under section 3.4.

Table 3.8 Combination of synthetic wastewater components in Plackett Burman Design

Run No.	Urea	NH_4Cl	CH_3COONa	Yeast	Peptone	Starch	KH_2PO_4	$FeSO_4$	$CuCl_2$	$MnSO_4$	Milk Powder
1	91.74	12.75	0	52.24	17.41	0	23.4	0	0	0	116.19
2	91.74	12.75	79.37	0	17.41	122	0	5.8	0	0	0
3	91.74	0	79.37	52.24	0	122	0	0	0	0.108	116.19
4	91.74	0	79.37	0	0	0	23.4	5.8	0.536	0	116.19
5	0	0	0	52.24	17.41	122	0	5.8	0.536	0	116.19
6	91.74	12.75	0	52.24	0	0	0	5.8	0.536	0.108	0
7	0	0	0	0	0	0	0	0	0	0	0
8	91.74	0	0	0	17.41	122	23.4	0	0.536	0.108	0
9	0	12.75	0	0	0	122	23.4	5.8	0	0.108	116.19
10	0	12.75	79.37	0	17.41	0	0	0	0.536	0.108	116.19
11	0	0	79.37	52.24	17.41	0	23.4	5.8	0	0.108	0
12	0	12.75	79.37	52.24	0	122	23.4	0	0.536	0	0

3.9 Photoinactivation of *E. coli* and *E. hirae* in synthetic wastewater using SAQS

Plackett Burman design of experiment was used to study the effect of synthetic wastewater components on bacterial photo-inactivation using SAQS. The components and their concentrations used are summarized in Table 7 and the experiments were designed using concentrations of the components as 0 and +1.

The experiments were conducted as described under section 3.8.1 except after dark incubation the suspension was exposed to a light intensity of 20 lux for 10 minutes using 6W UV-A, 9 inches phillips tube in a closed chamber. Duplicates of agar plate were then made by spreading 10 µl of photosensitized bacterial suspension and incubated at 37°C for 24 hours in the incubator. Viable cells in the culture plates were enumerated by colony counting method. The percentage inactivation of microorganisms from each duplicate runs in the study was calculated using equation 1 under section 3.4.

CHAPTER 4
RESULTS AND DISCUSSIONS

Water and wastewater use for irrigation and fertilization of agricultural lands raises concern about health hazards related to microbial contamination (Blumenthal et al., 2000; Kamyotra and Bhardwaj, 2011). In order to eliminate pathogenic germs, purification process includes a disinfection step. Disinfection is a chemical unit operation which commonly uses chlorine, hypochlorite, chloramines, chlorine dioxide, bromine and ozone (Acher et al., 1997). Among these, chlorine is the most widely used disinfectant for both drinking water and tertiary treatment of wastewater effluents (Gomes et al., 2009; Ergaieg and Seux, 2009 and Pablos et al., 2011). Its popularity is due to a high oxidizing potential which provides a minimum level of chlorine residual throughout the distribution system and protects against microbial recontamination (Sadiq and Rodrssiguez, 2004 and Gopal et al., 2007). However, it has now been realized that the disinfection by-products due to the chlorination process has both carcinogenic and mutagenic effects on mammals and, therefore, its validity has become questionable (Uyak and Toroz, 2005; Marugan et al., 2010 and Pablos et al., 2011).

Due to these reasons, more and more research is focused towards novel disinfection methods. Photosensitization currently appears to be promising among the various disinfection processes due to its easy implementation and cost effectiveness. It involves the combination of an active substance, i.e. a photosensitizer, light and molecular oxygen to achieve the purpose. Inactivation of microorganisms in water and wastewater using photo inactivating agents and the effect of organics present in wastewater is evaluated in this study.

This chapter discusses photo inactivation of *E. coli* and *E. hirae* using two individual photosensitive dyes, namely methylene blue and sodium anthraquinone-2- sulphonate. The chapter also discusses combined effect of these dyes on photo inactivation. It also includes the mechanism of photo inactivation and effect of synthetic wastewater components on photo inactivation.

4.1 Photoinactivation of *E. coli* and *E. hirae* in aqueous solution using Methylene Blue (MB)

Methylene blue (MB) is a water soluble cationic dye which falls under the class of phenothiazinium dyes. It absorbs in the range of 550 - 700 nm and has a high quantum yield. Hence the light source used for its activity in this study is a compact fluorescent light of 11W.

4.1.1 Effect of dark incubation time

Dark incubation prior to light exposure is required to achieve a homogeneous mixture of methylene blue and the bacterial cell suspension by keeping on a gel rocker for proper interaction. Initially this period was kept 5 minutes with constant shaking. The photo-inactivation efficiency achieved for *E. coli* and *E. hirae* varied in the range 10.41 - 29.20% and 10.81 - 30.97%, respectively (Fig. 4.1). Later, experiments were carried out with 15 minutes and 30 minutes dark incubation period to observe for any increase in photo-inactivation. The results obtained revealed an enhancement in the inactivation efficiency for both the bacteria due to methylene blue with the maximum efficiency obtained at 30 minutes dark incubation time. Photo-inactivation efficiency for *E. coli* and *E. hirae* varied in the range 11.62-43.53% and 11.74 - 44.66% (Fig. 4.2) respectively at 15 minutes of dark incubation whereas at 30 minutes of dark incubation the inactivation efficiency varied in the range 16.96 – 46.44% and 18.77 – 48.55% for *E. coli* and *E. hirae* respectively (Fig. 4.3).

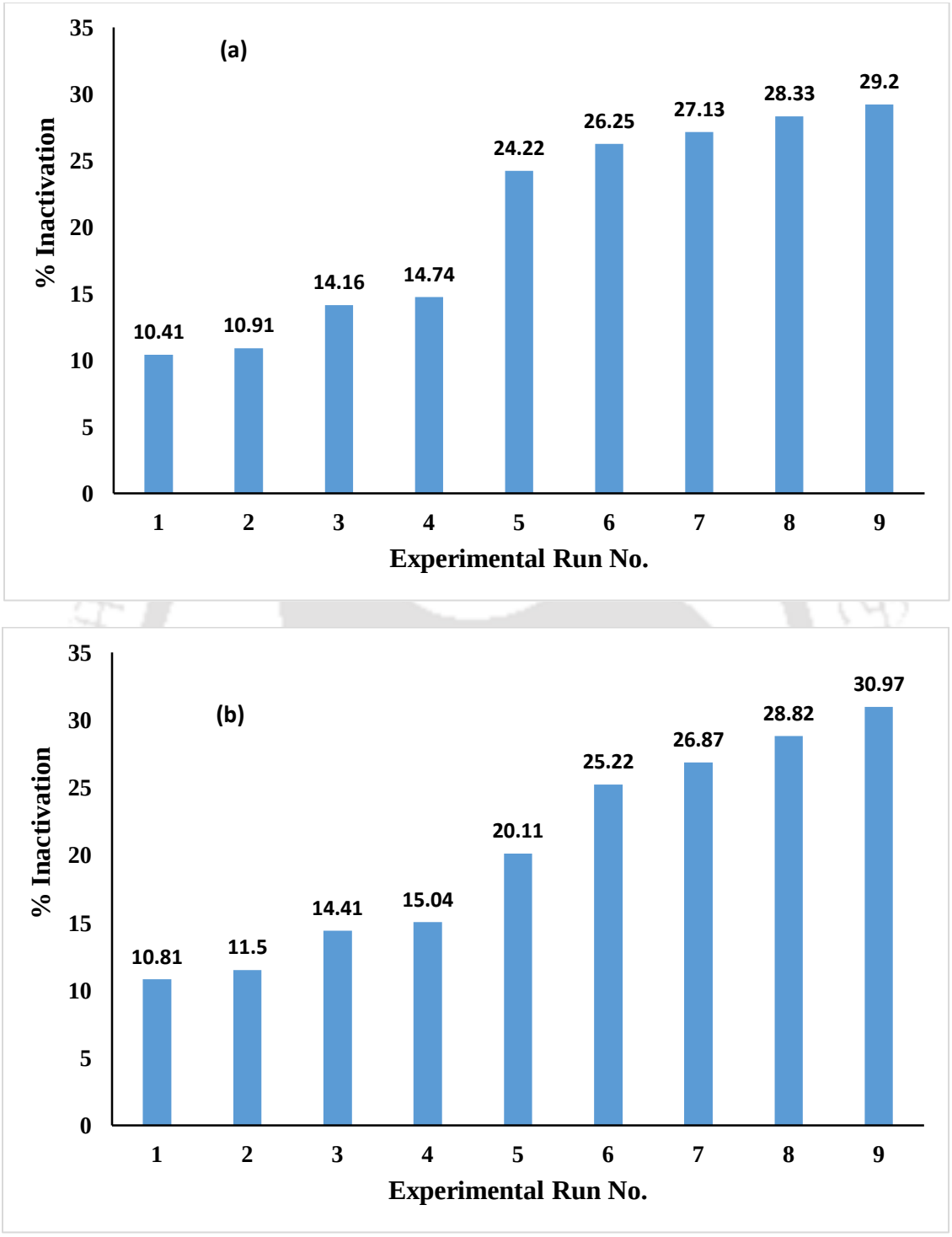


Fig. 4.1 Photo-inactivation obtained in the different experimental runs using MB (dark incubation period = 5 minutes) (a) *E. coli* (b) *E. hirae*

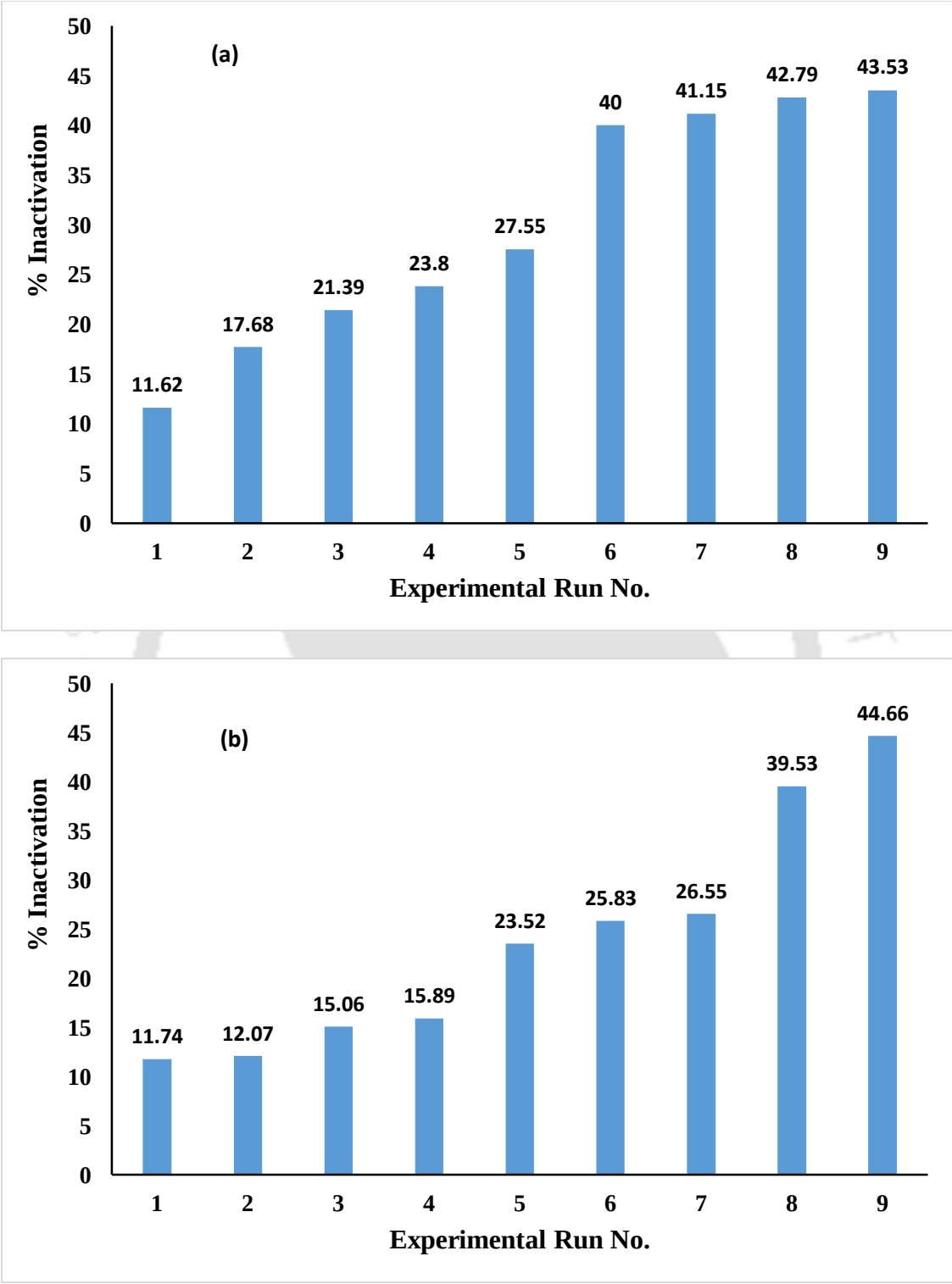


Fig. 4.2 Photo-inactivation obtained in the different experimental runs using MB (dark incubation period = 15 minutes) (a) *E. coli* (b) *E. hirae*

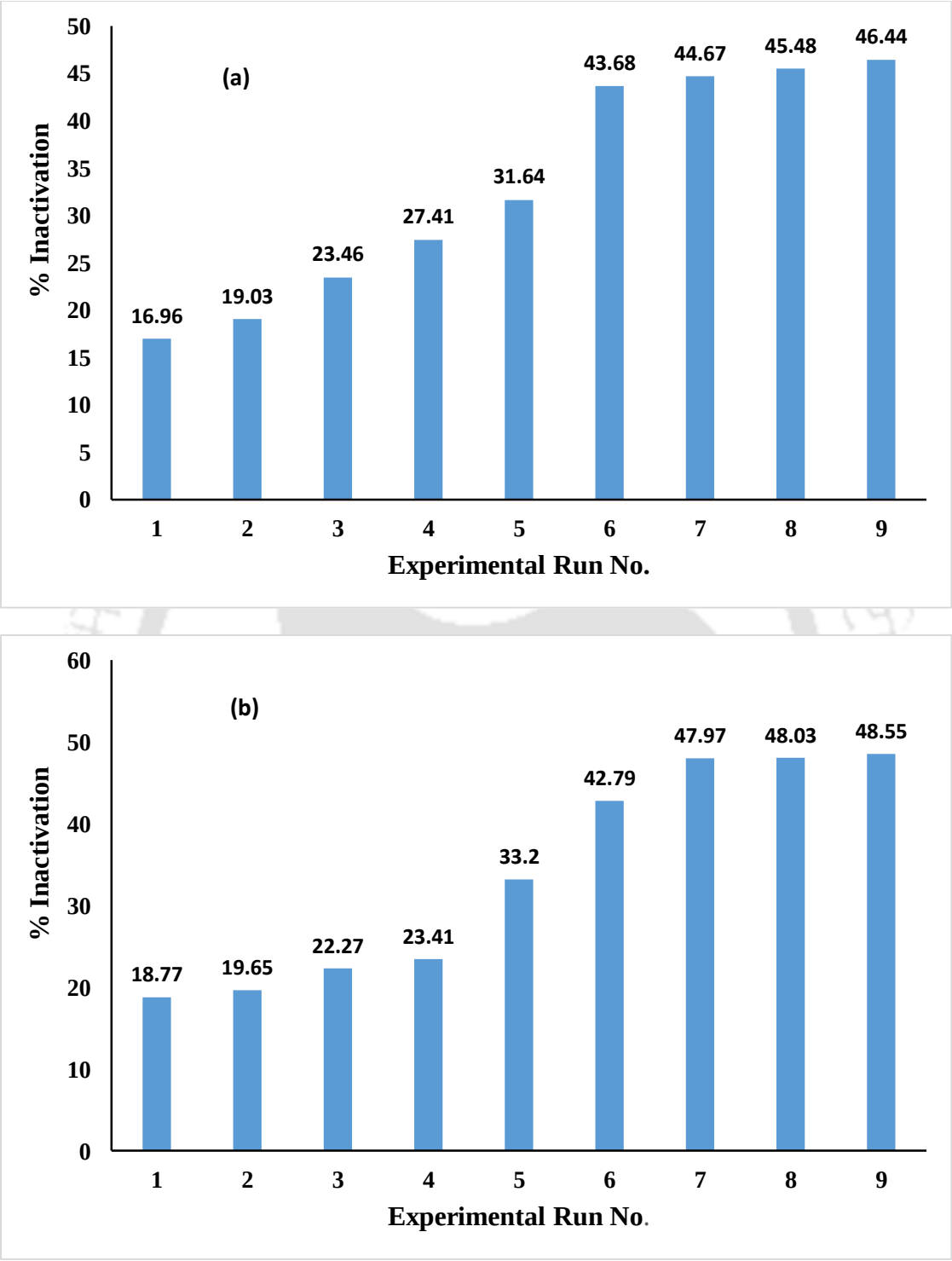


Fig. 4.3 Photo-inactivation obtained in the different experimental runs using MB (dark incubation period = 30 minutes) (a) *E. coli* (b) *E. hirae*

The enhanced photo-inactivation efficiency observed with an increase in the dark incubation period can be attributed to an increase in the contact time between the bacterial cells and MB at prolonged dark incubation, Ergaieg and Seux (2009) in their study suggest that positive charge on the photosensitizer molecule allows it to bind or in some cases penetrate into the microbial cell which is also in agreement with this study using the cationic dye MB.

4.1.2 Effect of initial photosensitizer concentration, Solution pH and initial viable cell count

The photo-inactivation efficiency of the bacteria increased with an increase in the PS concentration and a lower initial viable cell count of the bacteria (i.e. at higher dilutions). As increase in the pH of the cell suspension also showed significant effect on the photo-inactivation but depended on the combination levels of the other two factors initial MB concentration and initial viable cell count.

Initial MB concentration strongly influenced the photo-inactivation of both the microorganisms (Table 4.1 - 4.2), suggesting that the effect is due to either an increase in the quantum yield of reactive oxygen species (Ergaieg and Seux, 2009) or profound interaction of MB with bacterial surface or both (Jori and Brown, 2004). pH, on the other hand, showed a significant effect only at an alkaline pH and at a prolonged incubation period (30 minutes) in the dark. Chen et al., (2011) reported that MB is more effective under basic pH than under acidic condition probably due to its transition between singlet and triplet states. The pH effect was not significant for a short dark incubation period of 5 minutes probably due to insufficient contact time for interaction between the PS and the bacteria. Compared with initial MB concentration and pH, initial viable cell count is easily correlated to the bacterial inactivation. As the number of bacterial cells in the suspension decreases there is a chance of better interaction with MB at the same concentration as

compared with suspensions having high density of bacterial cells. Also, the chance of encountering reactive oxygen species increases when the initial viable cells are low, which further enhances the photo-inactivation efficiency of the bacteria. Similar results were reported in the literature but for photocatalysis of *E. coli* using TiO_2 (Benabbou et al., 2007).

Table 4.1 ANOVA of viable cell count at the end of the inactivation at 30 minutes dark incubation period with MB

	E. hirae						***E. coli***					
	F	P	R^2	R^2 Adj	SD	PRESS	F	P	R^2	R^2 Adj	SD	PRESS
Main effects	66.8	0.000	91.9	88.4	18.03	13134.1	169.8	0.000	96.7	95.2	12.5	6739.2
Two-way interaction effects	1.93	0.161					2.87	0.065				
Three-way interaction effects	0.24	0.632					0.21	0.652				

Table 4.2 Student '*t*' test of the regression coefficients of photo-inactivation of *E. hirae* and *E. coli* using MB

	E. hirae			***E. coli***		
Term	Coeff.	T	P	Coeff.	T	P
Constant	189.8	51.58	0.000	217.42	85.42	0.000
Conc.	-37.4	-10.16	0.000	-44.92	-17.65	0.000
pH	-4.29	-1.17	0.259	-9.58	-3.77	0.001
Dilution	36.04	9.80	0.000	34.50	13.55	0.000
Conc. and pH	0.87	0.24	0.815	3.25	1.28	0.218
Conc. and Dilution	-8.79	-2.39	0.028	-6.17	-2.42	0.026
pH and Dilution	0.62	0.17	0.867	-2.67	-1.05	0.309
Conc., pH and Dilution	1.79	0.49	0.632	1.17	0.46	0.652

4.1.3 Statistical analysis

For a better understanding of the role of different variables on the inactivation of *E. coli* and *E. hirae* using MB, statistical analysis of the results in the form of analysis of variance (ANOVA) and student't' test was performed.

In Table 4.1, which presents ANOVA of photo-inactivation results obtained at 30 min dark incubation period, the high Fischer's 'F' value and a low probability 'P' value of the regression model indicates its validity in explaining the variations in the results. Further, the results suggest that individual parameter effects and 2-way interaction effects due to the initial viable cell count were statistically significant. Accuracy and precision of the models, in the form of determination coefficient (R^2), adjusted R^2, standard deviation (SD) and predicted residual error sum of squares (PRESS) are shown in Table 4.1. All these results suggest that the models were highly efficient in predicting the experimental photo-inactivation results.

The estimated coefficients of individual and interaction effects between the variables, presented in Table 4.2, as well confirmed these results. Table 4.2 indicates a highly significant effect of initial MB concentration ($P<0.05$), initial viable cell count ($P<0.05$) and combined effect of MB initial concentration and initial viable cell count ($P<0.05$) on *E. hirae* inactivation using MB. In the case of *E. coli* inactivation using MB, significant effect is observed due to the MB concentration ($P<0.05$), pH of the suspension ($P<0.05$), initial viable cell count ($P<0.05$) and combined effect of initial MB concentration and initial viable cell count ($P<0.05$); however, the other interaction effects between the variables were found insignificant.

All these results of effect of variables on the photo-inactivation of *E. coli* and *E. hirae* are better depicted in the form of pareto charts, as illustrated in Fig. 4.4. Horizontal bars in these charts

represent effects (i.e. individual and interaction terms) of the parameters and the effects which extend past the reference line (vertical line on the chart) denote the significant ones (α = 0.05).

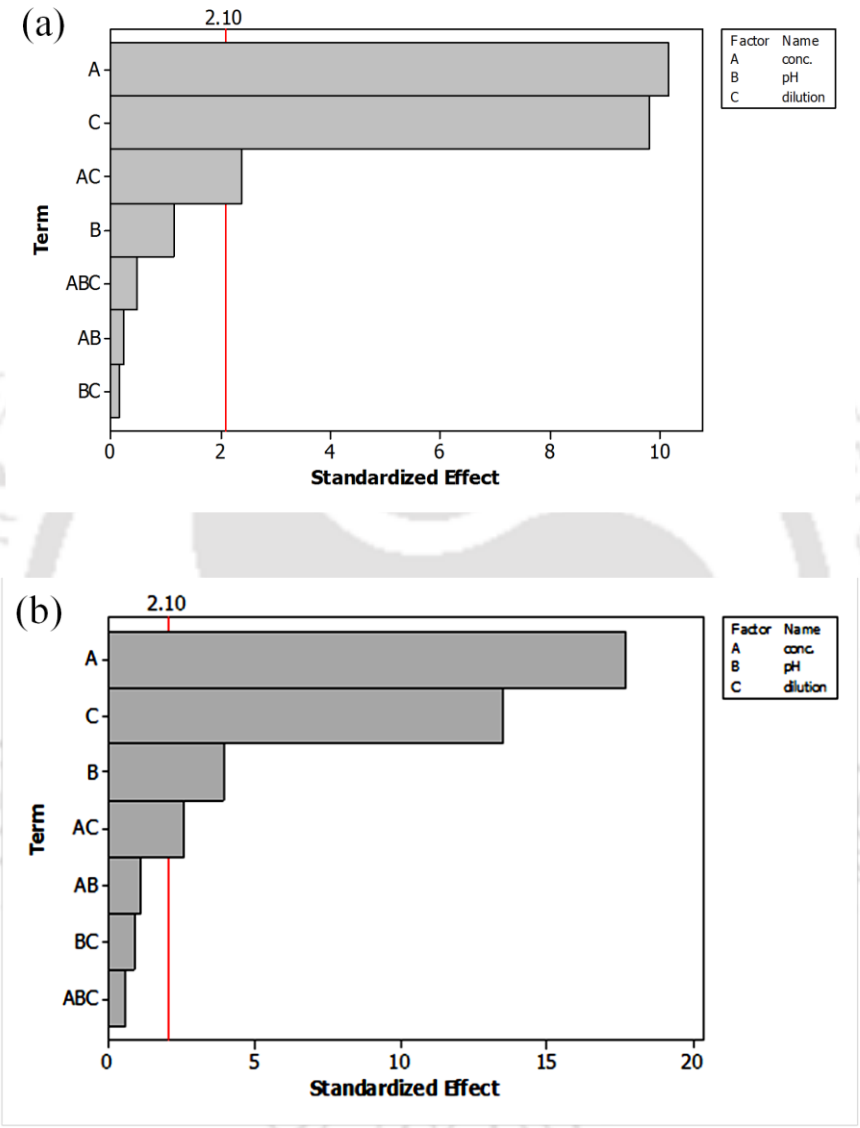


Fig. 4.4 Pareto chart showing the effect of different variables on photo-inactivation of bacteria using MB in presence of white light (a) *E. hirae* (b) *E. coli*

4.1.4 Mechanism of photoinactivation

The validity of the cell viability assay performed using the colony counting method was verified both qualitatively and quantitatively by flow cytometry (Fig. 4.5). As propidium iodide is not permeable through the intact cell membrane, it only gets internalized and binds to DNA of the cells whose membrane is compromised, hence this is used as the fluorescent probe to distinguish between live and dead cells (Amor et al., 2002) The shift in fluorescence peaks of propidium iodide is observed with an increase in the PS initial concentration for 30 min dark incubation time. This shift in the fluorescence peaks of propidium iodide added with PS treated bacteria from the standard peak represents the fact that the cell membrane in these bacteria was damaged thereby leading to their inactivation. In these histograms, gates M1 and M2 are defined in accord with the control sample (i.e bacterial suspension without treatment with MB) hence, the area under M_1 depicts the live cells whereas the area under M_2 represents dead cells. Statistical analyses of M_1 and M_2 (shown in Table 4.3) further revealed that photo-inactivation of *E. hirae* using MB is better than that of *E. coli*.

The results of ROS measurement (Fig. 4.6) revealed that the experimental run number 8 (as per the design in Table 3.2) yielded the maximum fluorescence in bacterial suspension and hence it can be concluded that maximum ROS is generated in this experimental run. Hence, there is a direct relationship between inactivation and ROS production. The results of lipid peroxidation and protein carbonyl index further suggest that the ROS generated during the reaction acts on the membrane of the bacteria and alters the lipid and protein of the membrane to form reactive aldehydes and/or ketones resulting in cell damage. The obtained results showed high lipid peroxidation and protein carbonylation (Table 4.4) levels for *E. hirae* as compared to *E. coli* when treated with MB (Yuichiro et al., 2010).

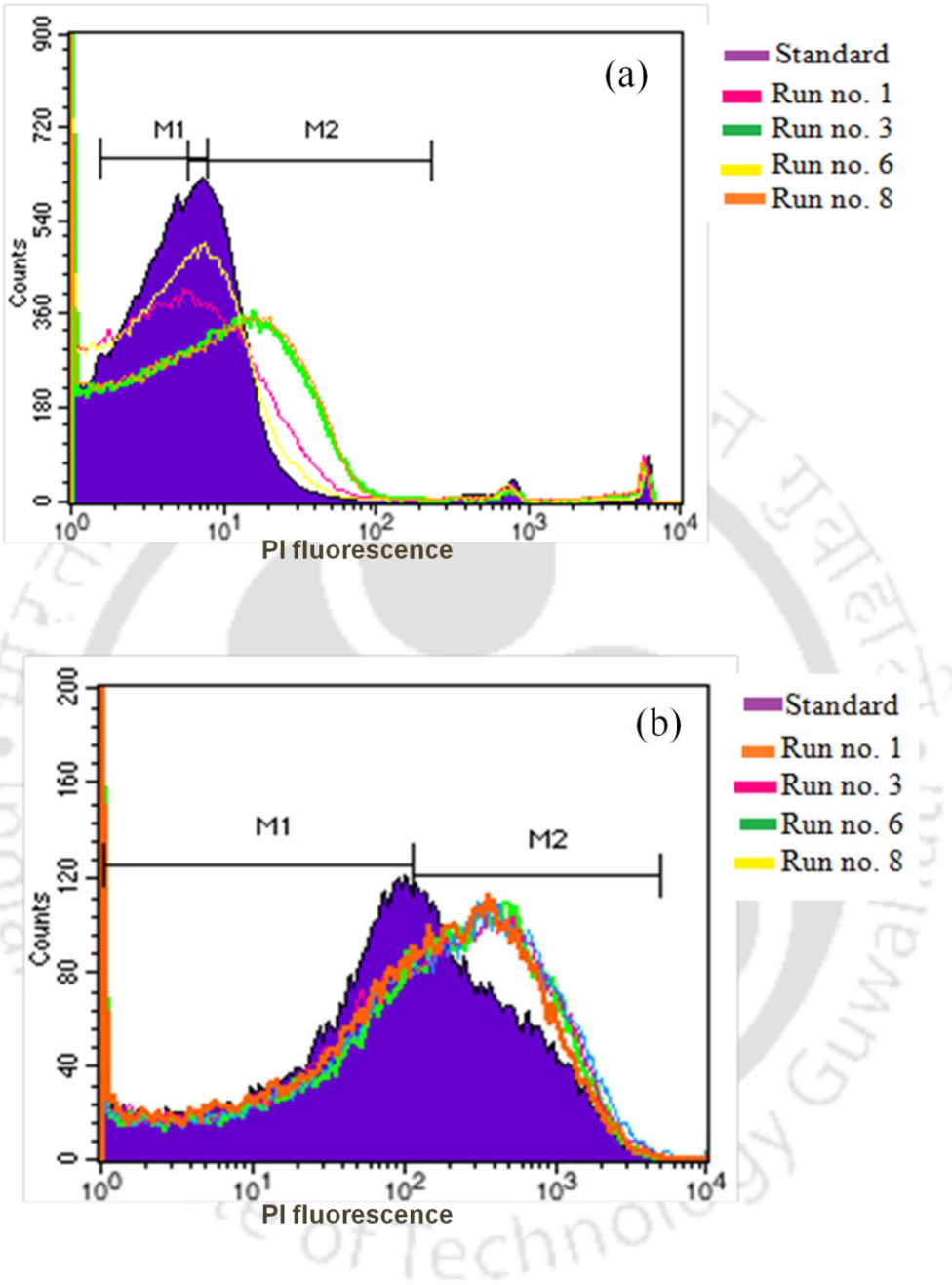


Fig. 4.5 Flow cytometry histograms obtained for photoinactivation using MB (a) *E. coli* and (b) *E. hirae* (dark incubation period = 30 minutes; Run 1,3,6 & 8 refers to Exp. Runs in Table 3.2)

These results are in accordance to the results obtained from flow cytometry analysis which proves that the alteration in the lipid and protein of the membrane causes cell damage. MB had showed more inactivation efficiency for Gram +ve *E. hirae* as compared to Gram –ve *E. coli*

which could be related to the presence of lipopolysaccharide membrane in *E. coli* which prevents efficient interaction between MB and the cell membrane.

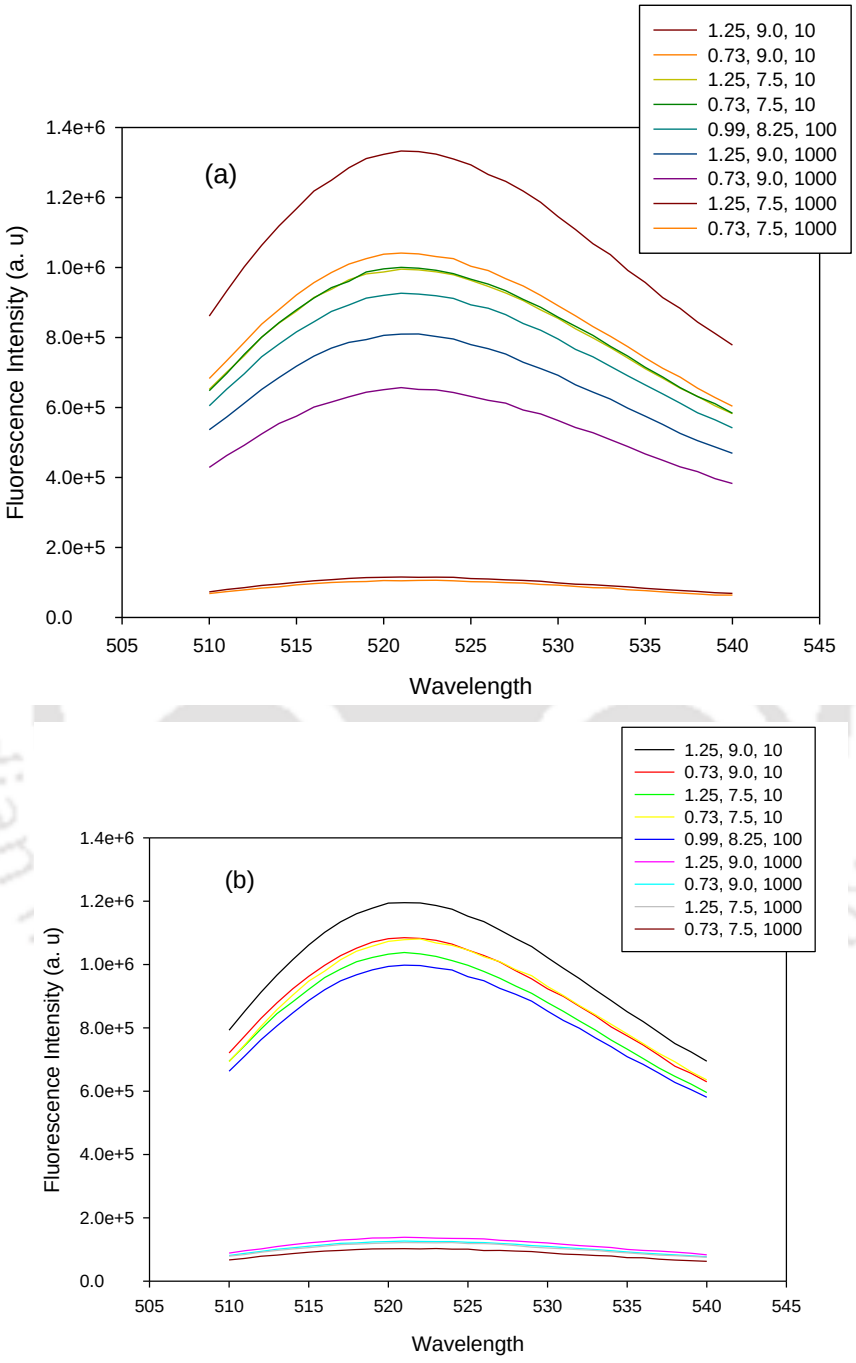


Fig. 4.6 Fluorescence curves for different experimental run (represented by combination of PS conc., pH and dilution in table 3.2) using MB (a) *E. hirae* (b) *E. coli*

Table 4.3 Percent live and dead cells obtained from the cell cytometric analysis data for photo-inactivation of bacteria using MB

	E. hirae		***E. coli***	
Run No.	M_1 %	M_2 %	M_1 %	M_2%
Standard	49.91	39.95	48.80	31.48
1	36.78	48.27	37.16	42.61
3	32.61	49.88	37.97	46.35
6	35.48	50.39	30.64	48.47
8	32.96	51.78	30.27	48.99

Table 4.4 Lipid peroxidation and protein carbonyl assay using MB

Process parameters		**Lipid peroxidation**		**Protein carbonylation**	
pH	Conc. of MB (μmol/l)	*E. coli*	*E. hirae*	*E. coli*	*E. hirae*
7.3	0	3.51	3.63	64.21	69.11
7.5	0.73	5.93	7.26	67.95	91.46
7.5	1.25	7.25	7.41	74.36	95.91
9.0	0.73	7.37	8.09	68.16	94.70
9.0	1.25	7.96	9.36	84.99	98.40

4.2 Photoinactivation of *E. coli* and *E. hirae* in aqueous solution using Sodium anthraquinone-2- sulphonate (SAQS)

Quinones are organic compounds which plays role in electron transport in bacteria, present in photosystem of plants etc. some quinones could act as photooxidizing agents of water in the presence of UV or visible light (Alegria et al., 1999). SAQS used in the following experiments is a water soluble, photostable, cationic dye which absorbs in UV-A region (Sun and Liu, 2002).

4.2.1 Effect of dark incubation time

Experiments on bacterial photo-inactivation using SAQS were carried out in the same manner as with those using MB except that the source of irradiation was 6W UV-A Philips tube light, of nine inches length and a light intensity of 20 lux. SAQS was added to the bacterial cell suspension and kept on a gel rocker in dark to achieve a homogeneous mixture and proper interaction between SAQS and bacterial cells. Dark incubation periods of 5, 15 and 30 minutes prior to illumination were studied.

The results reported that *E. coli* photo-inactivation efficiencies using SAQS was in the range 4.65 – 27.04% (Fig. 4.7a), 6.66 – 35.79% (Fig. 4.8a) and 10.90 – 37.66% (Fig. 4.9a) for 5, 15 and 30 minutes of dark incubation, respectively whereas, in case of *E. hirae* the inactivation efficiency was in the range 5.26 – 28.35% (Fig. 4.7b), 8.24 – 36.41% (Fig. 4.8b) and 10.03 – 39.03% (Fig. 4.9b) for 5, 15 and 30 minutes dark incubation period, respectively. A direct relationship between the dark period and inactivation efficiency was observed i.e. with increase in the dark incubation time an increase in photo-inactivation of bacterial cells occurred which could be due to the better interaction between the dye and bacterial cells (Ergaieg and Seux, 2009). The results of photoinactivation using SAQS are slightly lower than those observed using MB for both the organisms. This decrease in photo-inactivation observed can be attributed to the reduction in light intensity of UV-A tube light as compared to the visible light from the CFL, as all the other process parameters were constant.

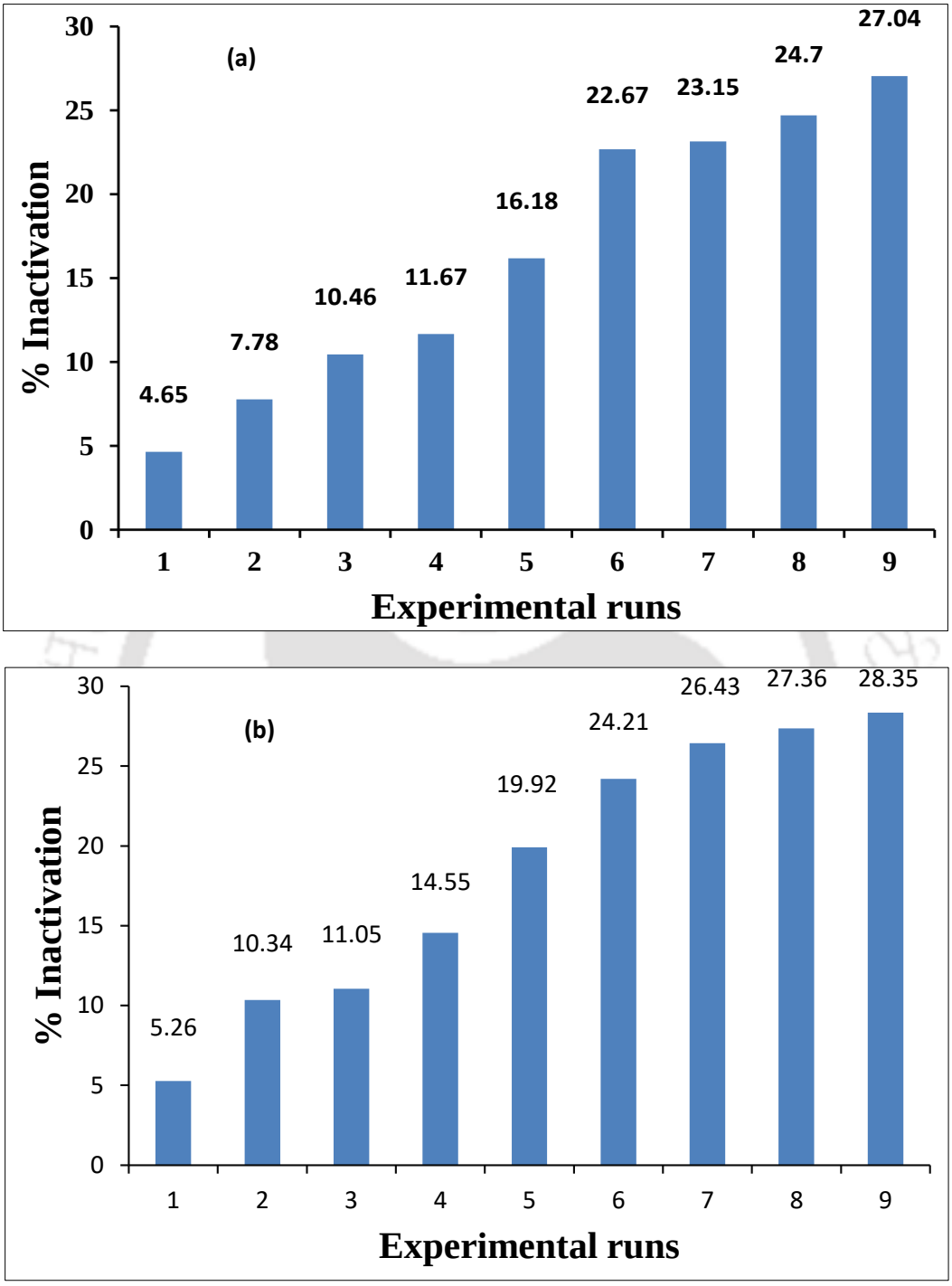


Fig. 4.7 Photo-inactivation obtained in the different experimental runs using SAQS (dark incubation period- 5 minutes) (a) *E. coli* (b) *E. hirae*

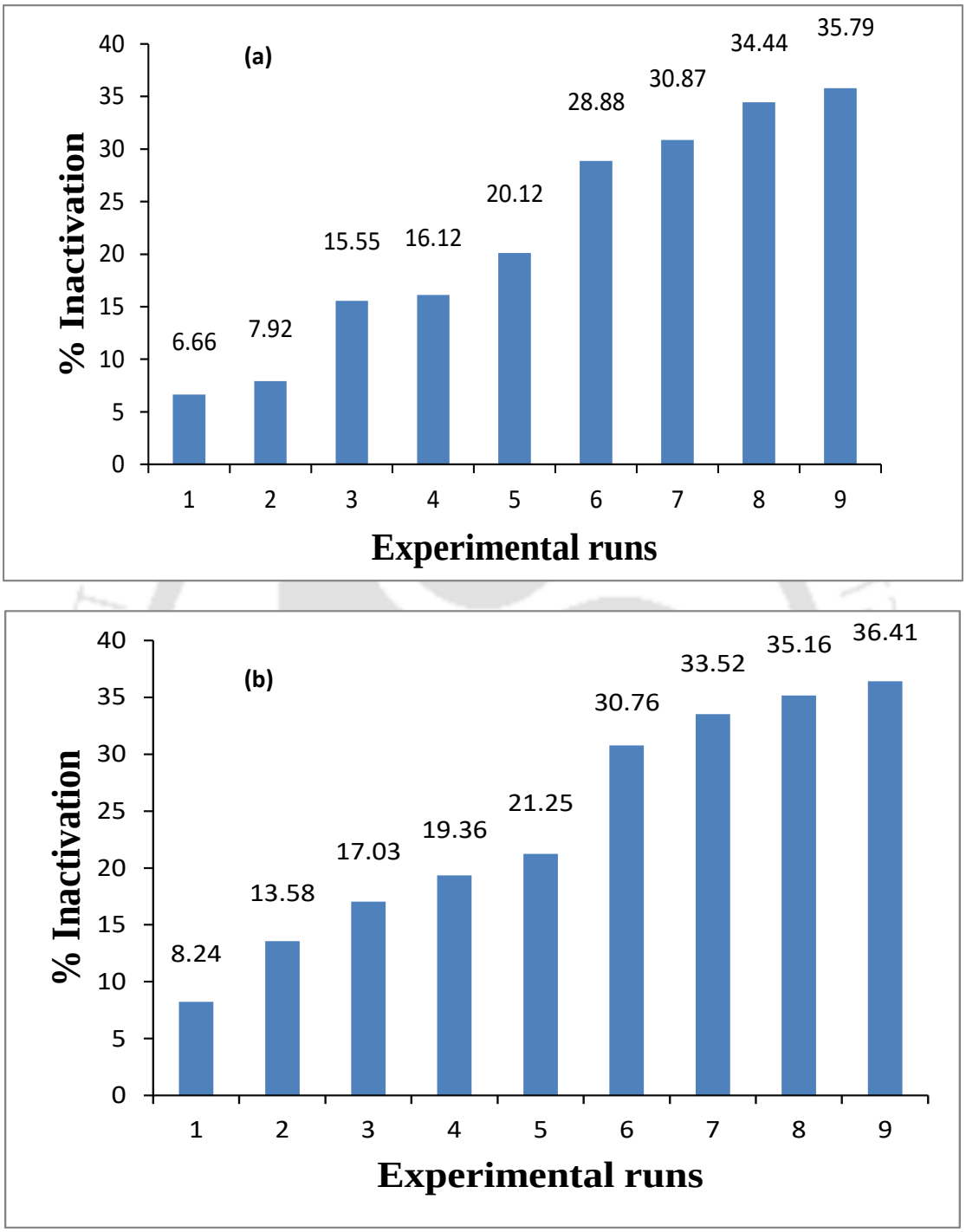


Fig. 4.8 Photo-inactivation obtained in the different experimental runs using SAQS (dark incubation period- 15 minutes) (a) *E. coli* (b) *E. hirae*

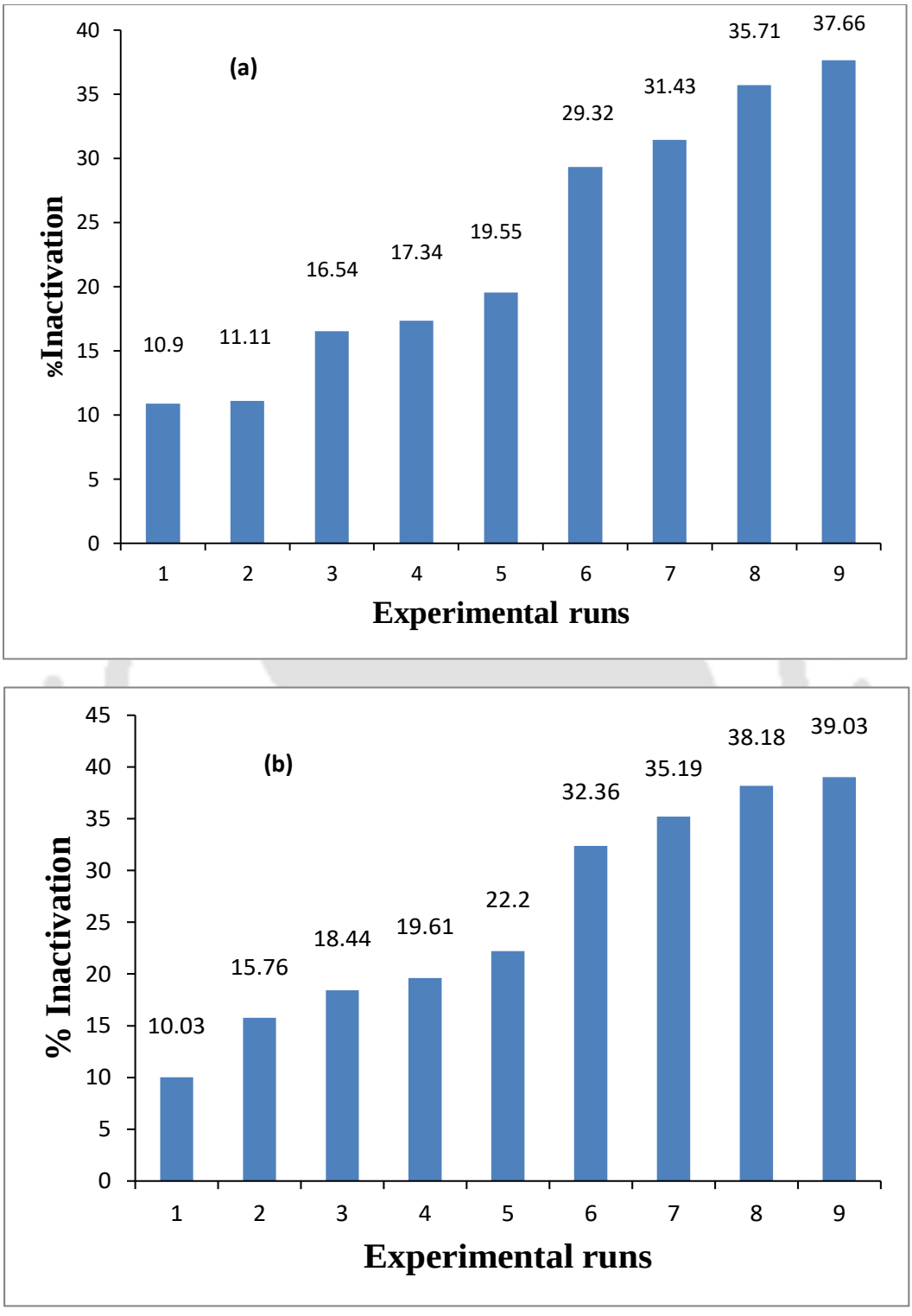


Fig. 4.9 Photo-inactivation obtained in the different experimental runs using SAQS (dark incubation period- 30 minutes) (a) *E. coli* (b) *E. hirae*

4.2.2 Effect of Photosensitizer Concentration, Solution pH and initial viable cell count

The parameters like photosensitizer concentration, solution pH and initial viable cell counts were kept same as in case of photo-inactivation using MB. Effect of parameters on photo-inactivation using SAQS were found in accordance with photo-inactivation involving MB. Results revealed increase in photo-inactivation efficiency with increase in concentration of photosensitizer and lower initial viable cell counts. The increase in solution pH also showed effect on inactivation efficiency but depended on the other two factors. Initial concentration of SAQS is found to strongly influence the photoinactivation of both the microorganisms which could be attributed to either the increase in quantum yield of reactive oxygen species (hydroxyl radical) or better interaction of SAQS with the bacterial surface or both (Jori and Brown, 2004). Increase in the solution pH showed increase in inactivation efficiency as higher pH could help quinone to achieve its excited state and help in production of hydroxyl radical (Chen et al., 2011 and Alegria et al., 1999). The correlation between initial viable cell count and bacterial inactivation could be easily understood as compared to initial SAQS concentration and solution pH. Decrease in the initial viable cell count increases the possibility of better interaction with SAQS and enhances the chance of encountering reactive oxygen species formed during the reaction. Hence, an increase in photoinactivation is observed with decrease in initial viable cell count (Benabbou et al., 2007).

4.2.3 Statistical analysis

To understand the role of different variables and their interation on the inactivation of *E. coli* and *E. hirae* using SAQS, statistical analysis of the results is done in the form of analysis of variance (ANOVA) and student't' test.

The ANOVA (Table 4.5) of photo-inactivation results obtained at 30 minutes dark incubation period with the high Fischer's 'F' value and a low probability 'P' value of the regression model indicates its validity in explaining the variations in the results. Accuracy and precision of the models, in the form of determination coefficient (R^2), adjusted R^2, standard deviation (SD) and predicted residual error sum of squares (PRESS), suggest that the models were highly efficient in predicting the experimental photo-inactivation results (Mahanty et al., 2010).

Table 4.5 ANOVA of viable cell count at the end of the inactivation at 30 minutes dark incubation period using SAQS

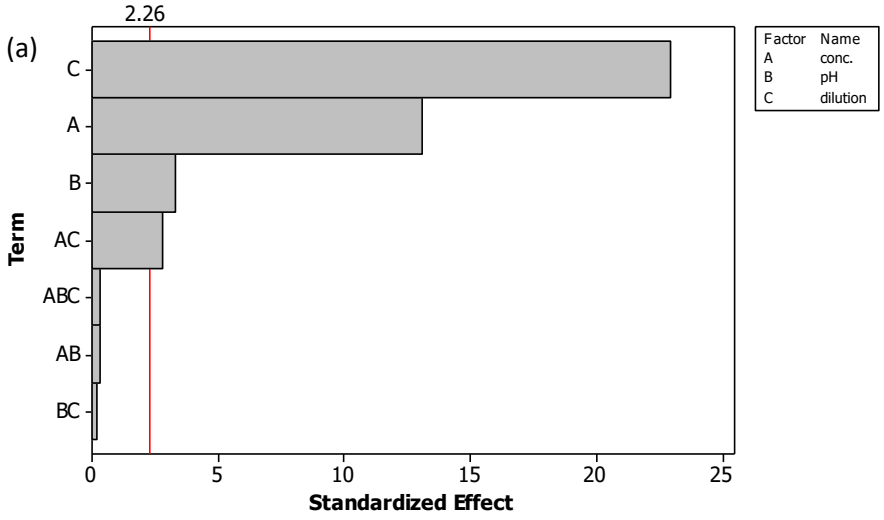
	E. hirae						***E. coli***					
	F	P	R^2	R^2 Adj	SD	PRESS	F	P	R^2	R^2 Adj	SD	PRESS
Main Effects	235.7	0.000	98.8	97.7	12.7	6381.69	153.50	0.000	98.13	96.5	9.24	3315.16
Two-way interaction effects	2.69	0.109					2.58	0.118				
Three-way interaction effects	0.11	0.745					0.01	0.937				

The estimated coefficients of individual and interaction effects between the variables, presented in Table 4.6, as well confirmed these results. It revealed significant effect of SAQS initial concentration ($P<0.001$), pH of the solution ($P = 0.009$ for *E. hirae* and $P = 0.002$ for *E. coli*), initial viable cell count ($P<0.001$) and combined effect of SAQS initial concentration and initial viable cell count ($P<0.05$) on the inactivation of *E. hirae* and *E. coli*.

Table 4.6 Student '*t*' test of the regression coefficients of photo-inactivation of *E. hirae* and *E. coli* using SAQS (dark incubation period = 30 minutes)

	E. hirae			***E. coli***		
Term	Coeff.	T	P	Coeff.	T	P
Constant	305.19	96.26	0.000	241.94	104.80	0.000
Conc.	-41.44	-13.07	0.000	-31.19	-13.51	0.000
pH	-10.44	-3.29	0.009	-9.94	-4.30	0.002
Dilution	72.69	22.93	0.000	37.19	16.11	0.000
Conc. and pH	0.94	0.30	0.774	-0.31	-0.14	0.895
Conc. and Dilution	-8.94	-2.82	0.020	-6.19	-2.68	0.025
pH and Dilution	0.56	0.18	0.863	-1.69	-0.73	0.483
Conc., pH and Dilution	-1.06	-0.34	0.745	0.19	0.08	0.937

Results on the effect of the three independent variables on photo-inactivation of *E. hirae* and *E. coli* are depicted in a better way in the form of pareto charts and are illustrated in Fig. 4.10 Horizontal bars in these charts represent effects (i.e. individual and interaction terms) of the parameters and the effects which extend past the reference line (vertical line on the chart) denote the significant ones ($\alpha = 0.05$).



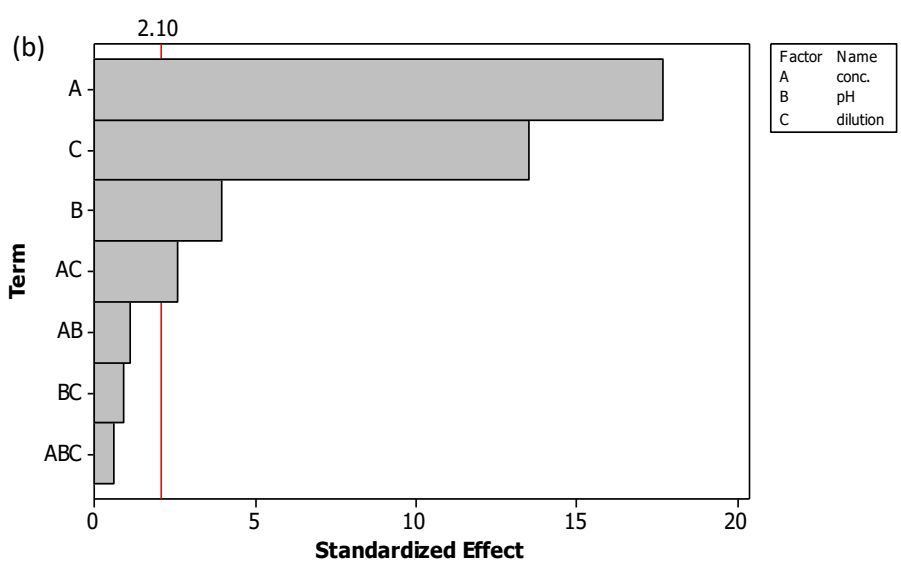


Fig 4.10 Pareto chart showing the effect of different variables on photo-inactivation of bacteria using SAQS in presence of UV-A light (a) *E. hirae* (b) *E. coli*

4.2.4 Mechanism of photoinactivation

Mechanism of photo-inactivation using SAQS was studied for better understanding of bacterial inactivation. Flow cytometric analysis of the treated cell sample was done to validate the results of colony counting method both qualitatively and quantitatively. Propidium iodide is known to get internalized and binds with the DNA of the cells whose membranes get compromised, hence could be used to distinguish between live and dead cells. The results (Fig. 4.11) showed a shift in the fluorescence peaks of propidium iodide with increase in SAQS concentration for 30 minutes dark incubation period. This shift in the fluorescence peaks of SAQS treated bacteria from the standard peak represents the fact that the cell membrane in these bacteria was damaged thereby leading to their inactivation. In accord to the control sample (i.e. bacterial suspension without treatment with SAQS) gates M_1 and M_2 are defined for these histograms where the area under M_1

depicts the live cells whereas area under M_2 represents dead cells Statistical analyses of M_1 and M_2 further revealed better photoinactivation of *E. hirae* using SAQS as compared to *E. coli* (Table 4.7).

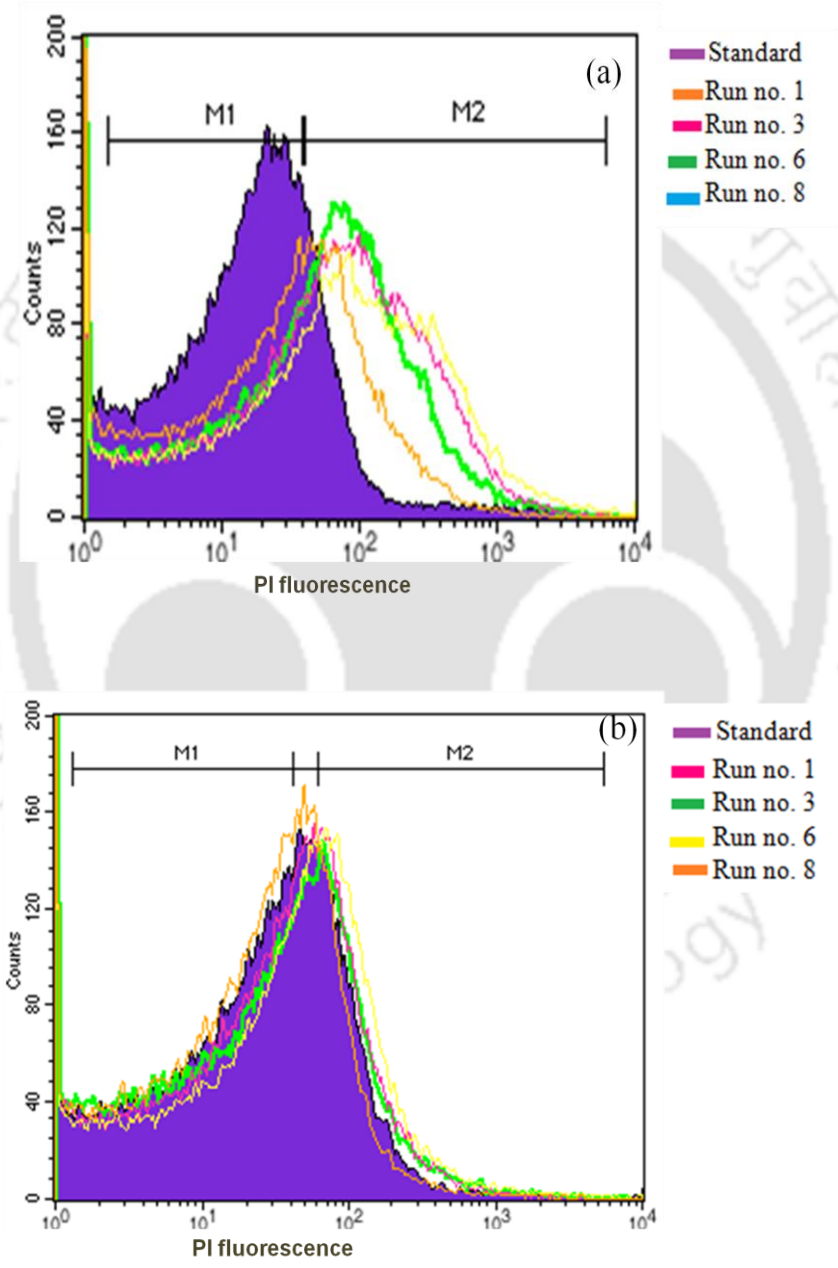


Fig. 4.11 Flow cytometry histograms obtained for photoinactivation using MB: (a) *E. coli* and (b) E. hirae (incubation period = 30 minutes; Run 1,3,6 & 8 refers to Exp. Runs in Table 3.2)

Table 4.7 Percent live and dead cells obtained from the cell cytometric analysis data for photo-inactivation of bacteria using SAQS

	E. hirae		***E. coli***	
Run No.	M_1 %	M_2 %	M_1 %	M_2 %
Standard	66.52	31.61	64.99	17.04
1	61.73	34.46	30.69	30.69
3	55.90	37.33	29.25	49.71
6	56.06	39.27	27.62	54.00
8	49.95	44.27	25.63	54.68

Table 4.8 Results of lipid peroxidation and protein carbonyl assay using SAQS

Process parameters		**Lipid peroxidation**		**Protein carbonylation**	
pH	Conc. of SAQS (μmol/l)	*E. coli*	*E. hirae*	*E. coli*	*E. hirae*
7.3	0	2.08	2.16	31.24	27.43
7.5	0.73	3.15	3.51	36.23	33.09
7.5	1.25	3.52	3.76	40.26	35.55
9.0	0.73	3.73	3.84	36.51	34.98
9.0	1.25	3.85	4.09	43.18	45.71

ROS measurement using Fluoromax 4 (Fig. 4.12) which confirmed generation of ROS on irradiation with maximum yield for run no. 8 (as per design Table 3.2). A direct relationship between inactivation and ROS production could be seen from colony counting method and fluoromax assay. The results of lipid peroxidation and protein carbonyl index (Table 4.8) which revealed that the changes are happening in the lipids and proteins of the membrane causing cell damage.

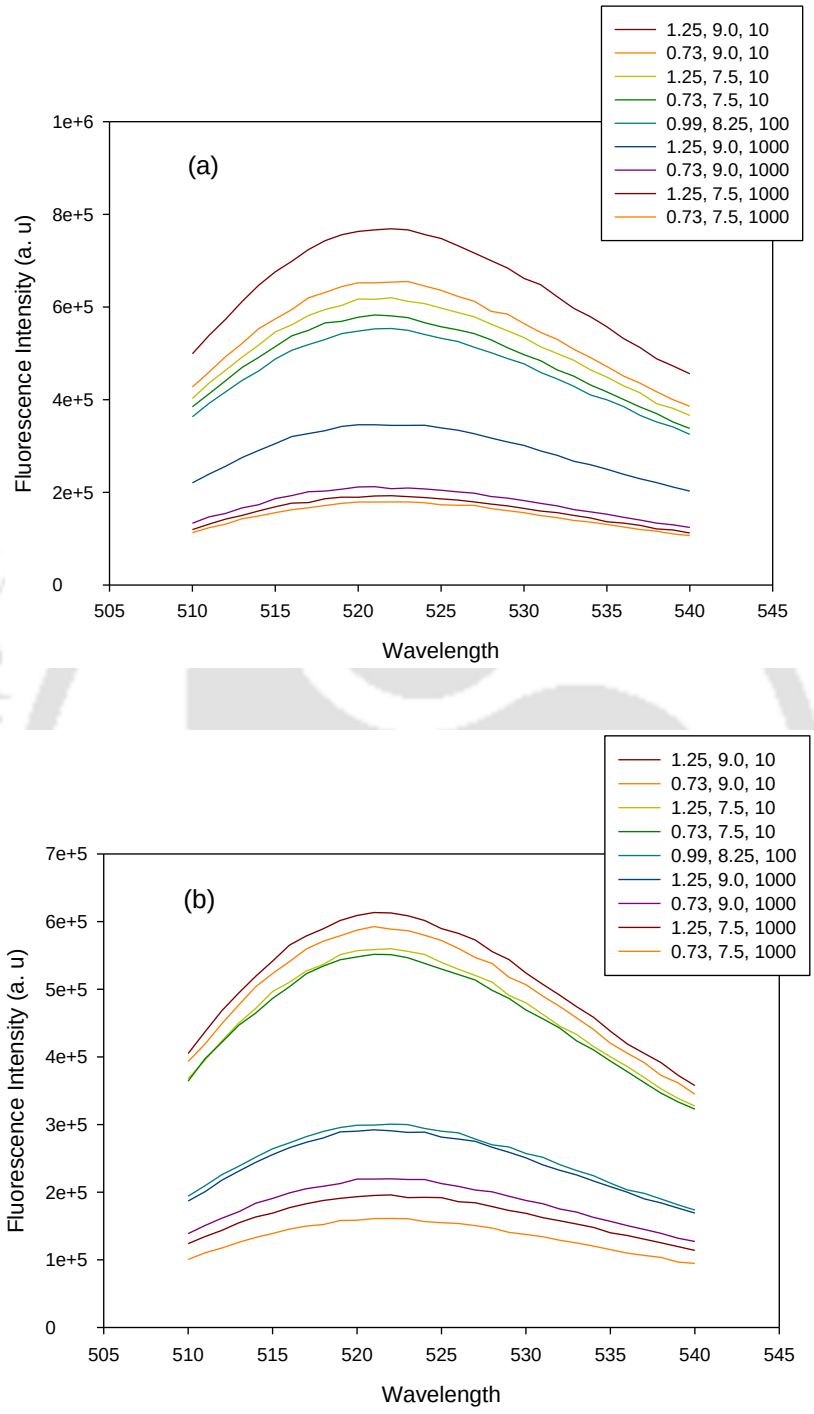


Fig. 4.12 Fluorescence curves for different experimental run (represented here by combination of PS conc., pH and dilution in table 3.2) using SAQS (a) *E. hirae* (b) *E. coli*

4.3 Photoinactivation of *E. coli* and *E. hirae* in aqueous solution using UV

The inactivation results of *E. coli* and *E. hirae* under UV illumination was evaluated by varying the UV intensity (energy), pH and dilution as shown in Table 3.5. The inactivation efficiency for *E. hirae* and *E. coli* varied in the range 28.40–99.66% and 13.17–99.56% respectively (Fig. 4.13). These values are higher as compared to the PS inactivation efficiency values.

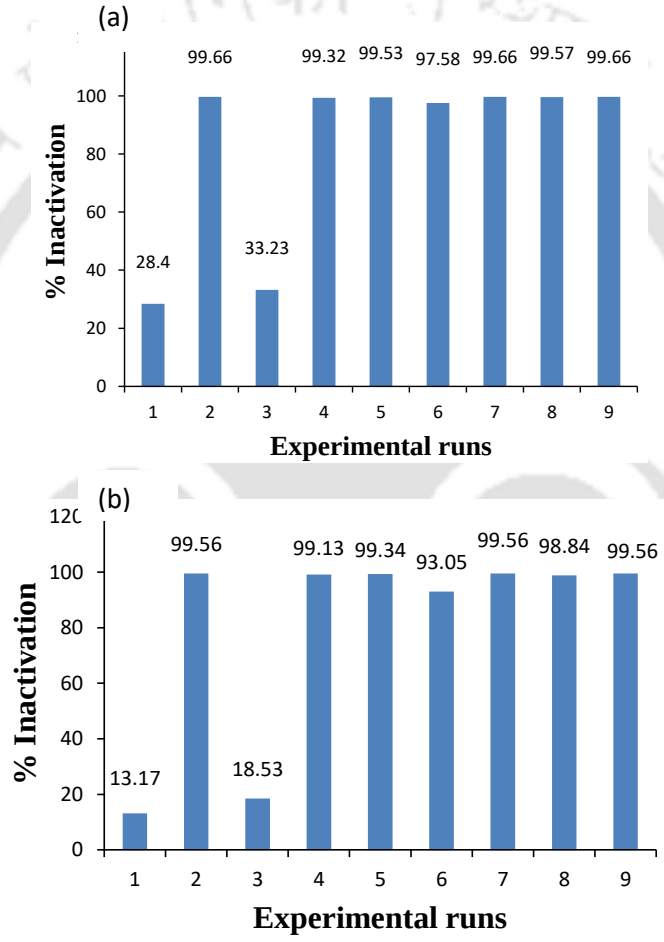


Fig. 4.13 Inactivation obtained in the different experimental runs using UV (a) *E. hirae* (b) *E. coli*

4.3.1 Statistical Analysis

The ANOVA (Table 4.9) of inactivation results with the high Fischer's 'F' value and a low probability 'P' value of the regression model indicates its validity in explaining the variations in

the results. Accuracy and precision of the models, in the form of determination coefficient (R^2), adjusted R^2, standard deviation (SD) and predicted residual error sum of squares (PRESS), suggest that the models were highly efficient in predicting the experimental photo-inactivation results (Mahanty et al., 2010).

Table 4.9 ANOVA of viable cell count at the end of the inactivation using UV

	E. hirae						***E. coli***					
	F	P	R^2	R^2 Adj	SD	PRESS	F	P	R^2	R^2 Adj	SD	PRESS
Main effects	416.49	0.000	99.53	99.12	9.718	11952.9	31.23	0.000	94.06	88.78	16.524	11725.8
Two-way interaction effects	200.93	0.000					14.45	0.001				
Three-way interaction effects	0.29	0.602					0.09	0.769				

Table 4.10 Student '*t*' test of the regression coefficients of photo-inactivation of *E. hirae* and *E. coli* using UV

	E. hirae			***E. coli***		
Term	Coeff.	T	P	Coeff.	T	P
Constant	62.81	25.85	0.000	30.38	7.35	0.000
Energy	-59.81	-24.62	0.000	-27.25	-6.60	0.000
pH	-2.81	-1.16	0.277	-2.88	-0.70	0.504
Dilution	-61.56	-25.34	0.000	-29.13	-7.05	0.000
Energy and pH	1.06	0.44	0.672	1.00	0.24	0.814
Energy and Dilution	59.56	24.52	0.000	27.00	6.54	0.000
pH and Dilution	3.06	1.26	0.239	3.13	0.76	0.469
Energy, pH and Dilution	-1.31	-0.54	0.602	-1.25	-0.30	0.769

The estimated coefficients of individual and interaction effects between the variables, presented in Table 4.10, as well confirmed these results. It revealed significant effect of UV energy ($P<0.001$), initial viable cell count ($P<0.001$) and combined effect of UV energy and initial

viable cell count (P<0.05) on the inactivation of *E. hirae* and *E. coli*. Results on the effect of the three independent variables on inactivation of *E. hirae* and *E. coli* are depicted in a better way in the form of pareto charts and are illustrated in Fig. 4.14 Horizontal bars in these charts represent effects (i.e. individual and interaction terms) of the parameters and the effects which extend past the reference line (vertical line on the chart) denote the significant ones (α = 0.05).

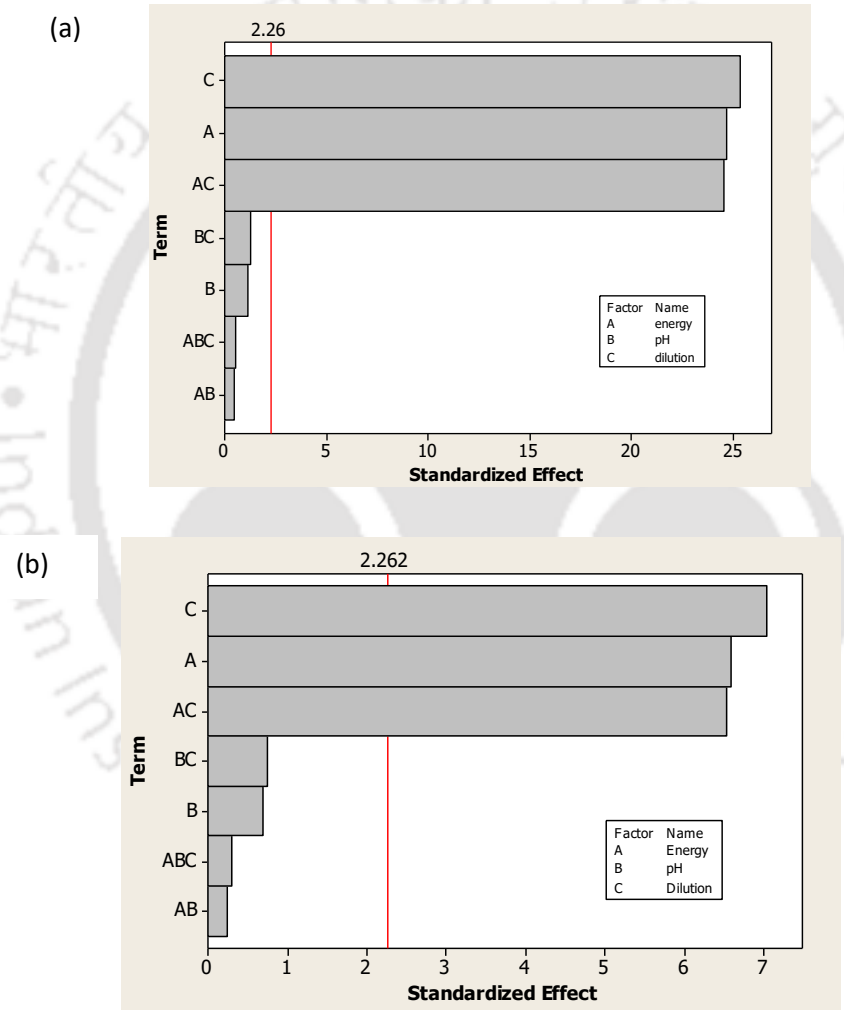


Fig 4.14 Pareto chart showing the effect of different variables on photo-inactivation of bacteria using UV (a) *E. hirae* (b) *E. coli*

4.4 Combined effect of MB and SAQS on photo-inactivation of *E. coli* and *E. hirae*

Experiments involving the individual photosensitizers (MB and SAQS) on photo-inactivation of *E. coli* and *E. hirae* revealed that a combination of 30 minutes dark incubation period, lower initial viable cell count and alkaline pH of the solution is effective for a maximum inactivation efficiency. As individually both the dyes have shown significant effect against inactivation of both the microorganisms, hence, to check their effect on inactivation of the microorganisms when added together to the bacterial suspension, the present experiment was designed according to the statistically valid full factorial design as described under section 3.7. The other process parameters were selected from the previous study for giving best inactivation results i.e. pH=9.0 and low initial viable count (1000 times diluted cell suspension) were used. The light source used in this study was a closed chamber fitted with four tube lights of 6W each. Out of which two were tube lights emitting in the visible region and the other two were emitting in the UV-A region.

Photo inactivation efficiency of MB and SAQS in combination and at pH 9.0 varied in the range of 27.02% - 47.09% for *E. coli* and in the range of 32.87% - 49.50% for *E. hirae* (Fig. 4.15). A large variation in the inactivation efficiency range of *E. coli* and *E. hirae* is observed, which could be attributed to the combination of dyes (i.e. -1&-1 or +1 & +1 etc.) as well as to the lipopolysaccharide membrane present in Gram –ve *E. coli* which prevents interaction of dyes with the cell membrane. An increase (1.5 to 2%) in inactivation efficiencies of PS (MB and SAQS) is observed in case of both the microorganisms when compared with individual inactivation efficiencies of the PS which yielded maximum 48.55% inactivation for E. hirae and 46.44% inactivation for E. coli with MB and 39.03% inactivation for E. hirae and 37.66% inactivation for E. coli with SAQS.

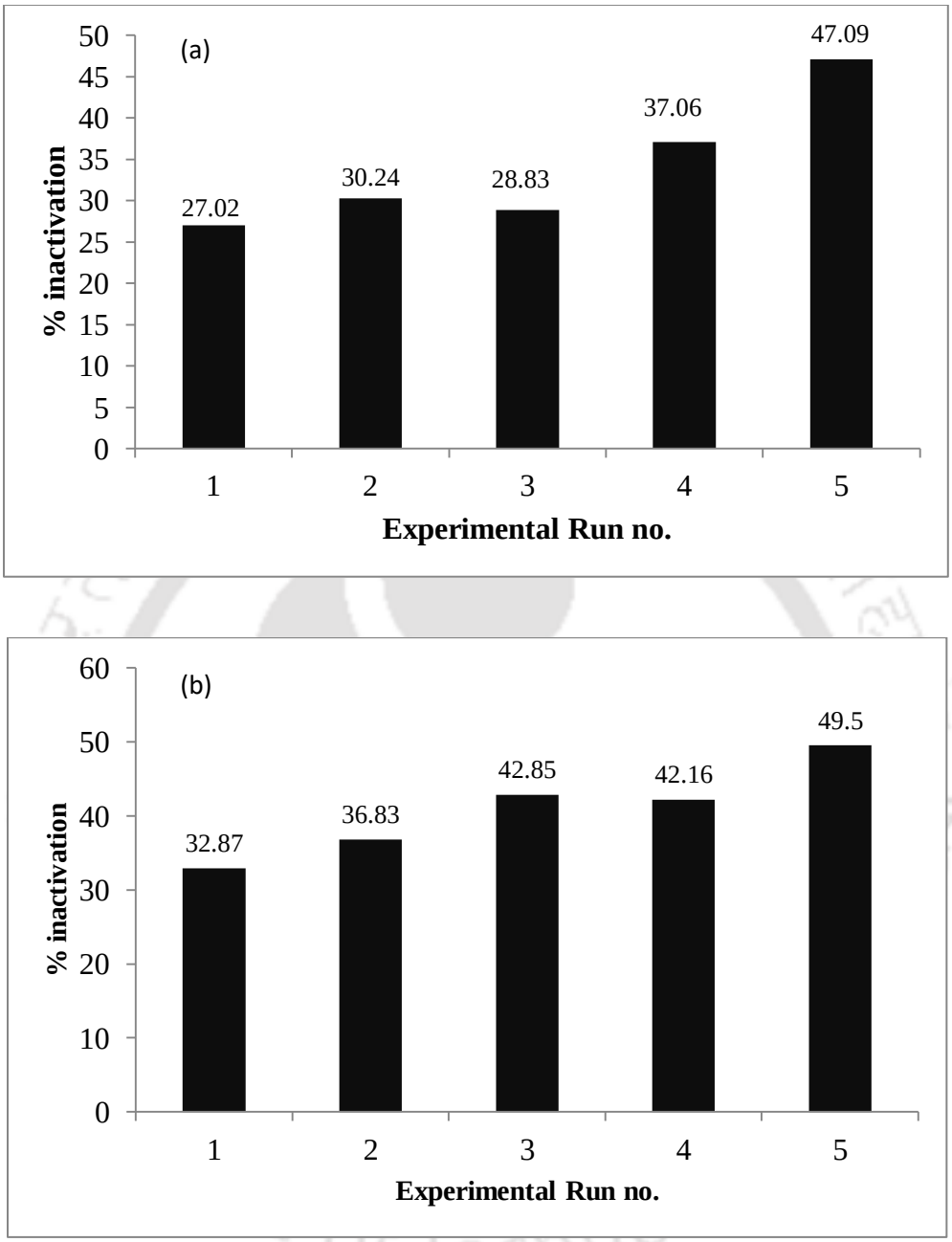


Fig. 4.15 Photo-inactivation of *E. hirae* and *E. coli* in the different experimental runs using MB and SAQS together, (a) *E. coli* and (b) *E. hirae* (pH = 9.0, 1000 dilutions and 30 minutes dark incubation period)

This increase in inactivation efficiency observed can be attributed to the increase in reactive oxygen species (ROS) production due to the presence of both the dyes as well as the light sources for their activation. MB and SAQS are known to produce ROS when irradiated with light of appropriate wavelength and inactivate microorganisms (Tuite and Kelly, 1993 and Novotny et.al, 1998) as also observed in our previous study with individual dyes. It was expected that the inactivation efficiency will increase significantly when both the PS are mixed together, as compared to individual PS and could be equal to the cumulative efficiency by individual PS whereas the observed results were only slightly higher (1.5% - 2%) than with individual PS. The low increase in inactivation may be due to the competition for the available molecular oxygen for production of ROS. Both the PS were added to the same 15 ml bacterial suspension so the total concentration of PS in each sample was double the concentration when added individually in previous study. The duration of light is kept constant for 10 minutes but the intensity has decreased from 2700 lux to 1500 lux, the insufficient light may be a cause for decrease in inactivation efficiency (Prates et al., 2009).

4.4.1 Statistical Analysis

For a better understanding of the combined PS effect on the inactivation of these two bacteria statistical analysis of the results in the form of analysis of variance (ANOVA) and student ‘t’ test was performed.

In Table 4.11, which presents ANOVA of photo-inactivation results obtained for combined effect of PS, the high Fischer’s ‘F’ value (9.86) and a low probability ‘P’ value (0.004) of the regression model indicates its validity in explaining the variations in the results. Further, the results suggest that individually MB has a significant effect. Accuracy and precision of the

models, in the form of determination coefficient (R^2= 67.69 and 29.58 for *E. hirae* and *E. coli* respectively), adjusted R^2 (54.76 and 1.41 for *E. hirae* and *E. coli* respectively), standard deviation (SD= 24.90 and 37.42 for *E. hirae* and *E. coli* respectively) and predicted residual error sum of squares (PRESS= 13578.4 and 29511.6 for *E. hirae* and *E. coli* respectively) shown in Table 11a and 11b, suggest that the models were average in predicting the experimental photo-inactivation results.

Table 4.11 ANOVA of viable cell count at the end of the inactivation at 30 minutes dark incubation period with MB and SAQS (a) *E. hirae* (b) *E. coli*

(a)	***E. hirae***					
	F	**P**	**R^2**	**R^2 Adj**	**SD**	**PRESS**
Main effects	9.86	0.004	67.69	54.76	24.901	13578.4
Two-way interaction effects	0.09	0.769				

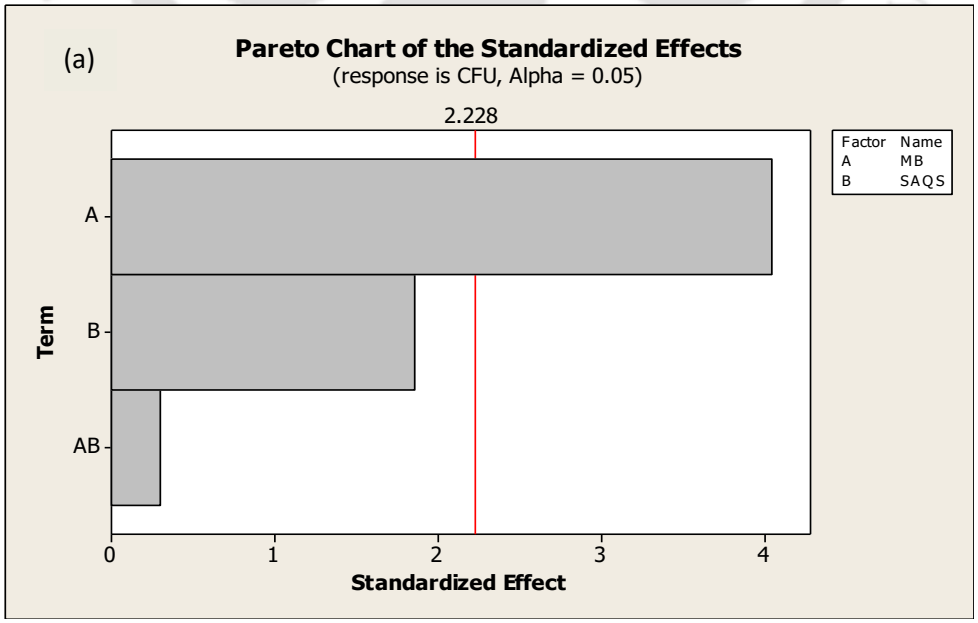
(b)	***E. coli***					
	F	**P**	**R^2**	**R^2 Adj**	**SD**	**PRESS**
Main effects	1.68	0.236	29.58	1.41	37.424	29511.6
Two-way interaction effects	0.24	0.637				

The estimated coefficients of individual and interaction effects between the variables, presented in Table 4.12, as well confirmed these results. Results indicate a highly significant effect of MB concentration (P<0.05) for *E. hirae* but no significant effect for *E. coli* inactivation however, the other individual and interaction effects were found insignificant.

Table 4.12 Student '*t*' test of the regression coefficients of photo-inactivation of *E. hirae* and *E. coli* using MB and SAQS together

	E. hirae			***E. coli***		
Term	Coeff.	T	P	Coeff.	T	P
Constant	7.188	48.67	0.000	10.80	30.99	0.000
MB	7.188	-4.03	0.002	10.80	-1.81	0.100
SAQS	7.188	-1.85	0.093	10.80	-0.25	0.804
MB & SAQS	7.188	-0.30	0.769	10.80	0.49	0.637

All these results about effect of PS on the photo-inactivation of *E. hirae* and *E.coli* are depicted in a better way in the form of pareto charts (Fig. 4.16). Horizontal bars in these charts represent effects (i.e. individual and interaction terms) of the parameters and the effects which extend past the reference line (vertical line on the chart) denote the significant ones (α = 0.05).



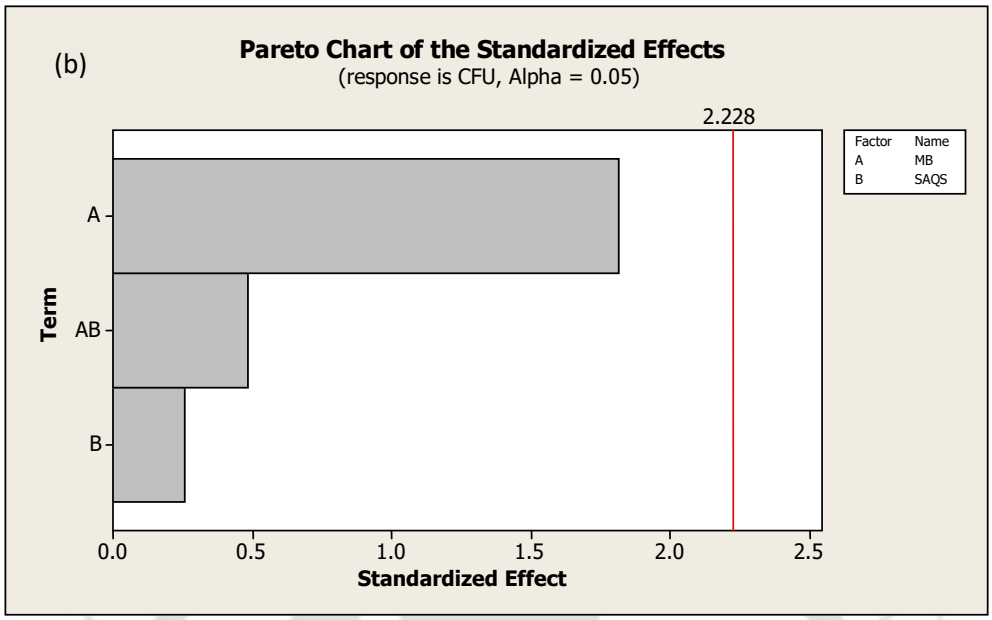
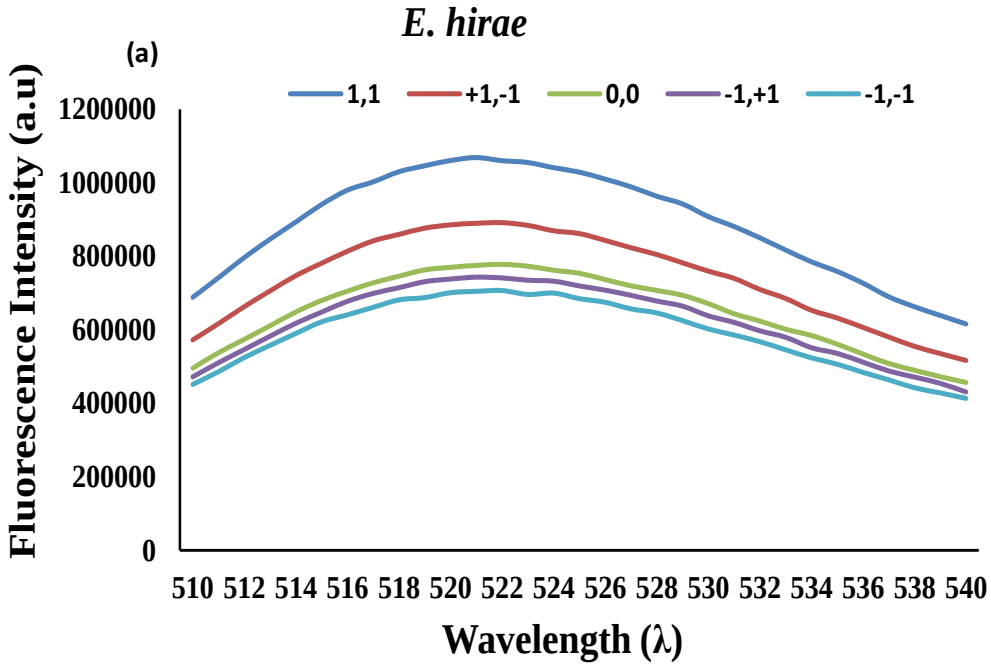


Fig 4.16 Pareto chart showing the effect of MB and SAQS on photo-inactivation of (a) *E. hirae* (b) *E. coli*



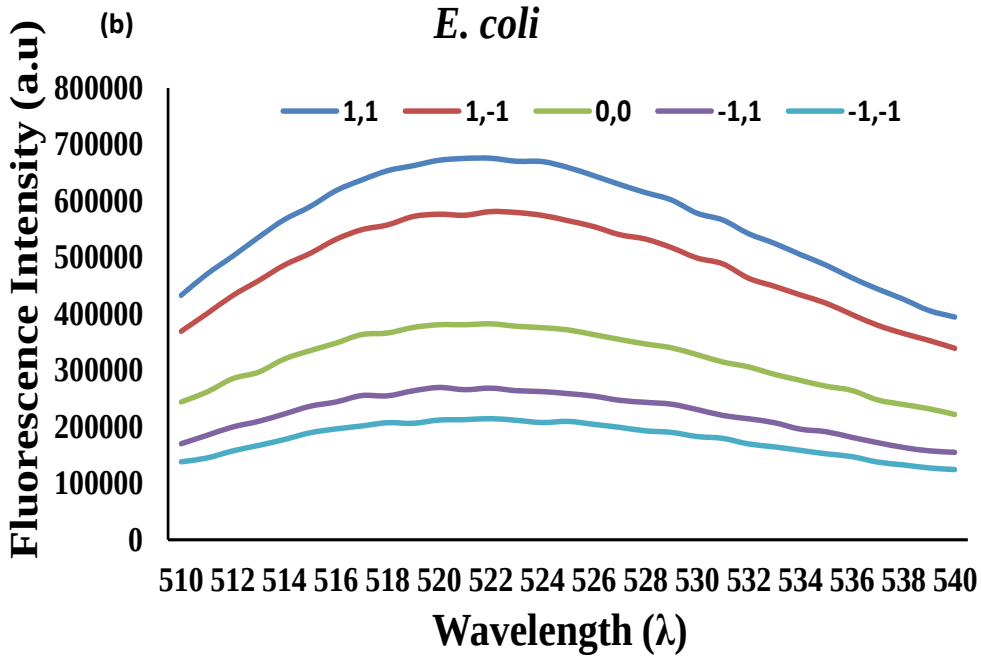


Fig. 4.17 Fluorescence curves for different experimental run with both the dyes (a) *E. hirae* (b) *E. coli*

ROS is measured as explained under section 3.5 and the results revealed that experimental run with maximum concentration of both the dyes gives (Fig. 4.17) the maximum fluorescence in bacterial suspension and hence it can be concluded that maximum ROS is generated in this experimental run. ROS data when (Fig. 4.17) compared to viable cell count data (Fig. 4.15) shows direct relationship between inactivation and ROS production.

4.5 Effect of synthetic wastewater components on photo-inactivation

Experiments were conducted previously on bacterial photoinactivation using MB and SAQS individually in aqueous cell suspension, illuminated by white light and UV-A light respectively. The maximum inactivation efficiencies obtained were 46.44% for *E. coli* and 48.55% for *E. hirae* using MB whereas using SAQS the maximum efficiencies were 37.66% for *E. coli* and

39.03% for *E. hirae* at 30 minutes dark incubation period. Statistical analysis of results revealed a significant effect of photosensitizer concentration, solution pH and initial viable cell counts along with some significant interaction effects between the process parameters. From this study we deduced that maximum photoinactivation takes place at high concentration of photosensitizer, alkaline pH and low initial viable cell counts in the suspension. To simulate the real wastewater conditions and see the effect on photoinactivation efficiency of MB and SAQS as well the effect of wastewater components, the current experiment was conducted in municipal synthetic wastewater (MSWW). The components of the MSWW used are described in Table 3.7 and the process parameters for this experiment were chosen as concentration of photosensitizer = 1.25µmol/l, pH of solution = 9.0 and low initial viable cell count (i.e. dilution = 1000 times).

The experiment was designed according to the Plackett Burman design and the concentration of the components were taken as 0 and +1. To maintain the final concentration of the MSWW components in the final sample volume (15ml) of the cell suspension a stock of concentration 1M was made for each component and 15µl of the stock was added in the sample volume before adding MB or SAQS. The MSWW components added to each sample tube were according to the combination of MSWW components described in each run nos. as mentioned in Table 3.8.

4.5.1 Photo-inactivation using MB and SAQS individually

Photo-inactivation efficiency for *E. hirae* and *E. coli* varied in the range 10.9 – 48 % and 9.9 – 46 % (Fig. 4.18) respectively using MB whereas in case of SAQS the inactivation efficiency varied in the range 8.89 – 39 % and 8.55 – 37 % (Fig. 4.19) for *E. hirae* and *E. coli* respectively.

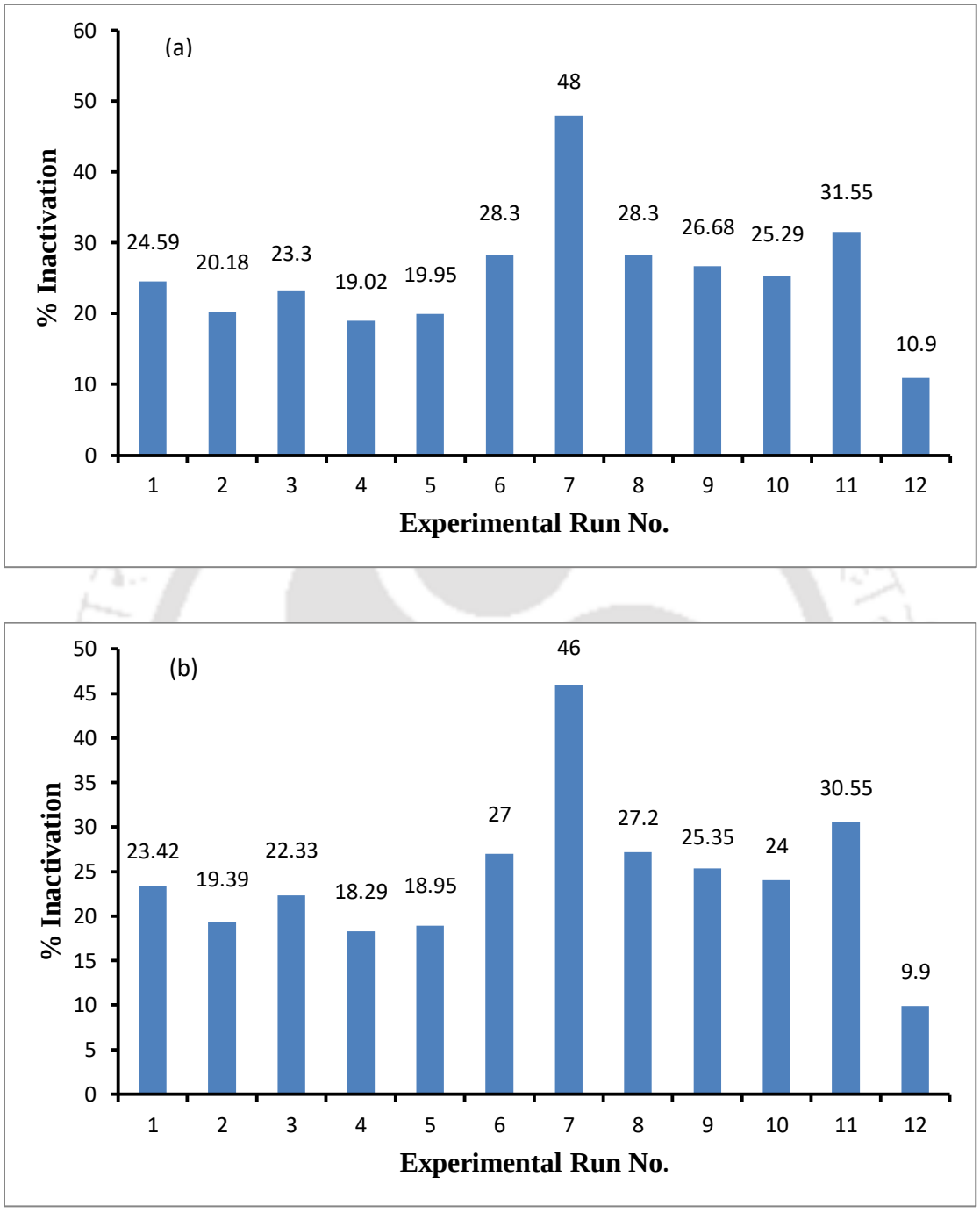


Fig. 4.18 Photo-inactivation obtained in the different experimental runs using MB for 30 minutes dark incubation period (a) *E. hirae* (b) *E. coli*

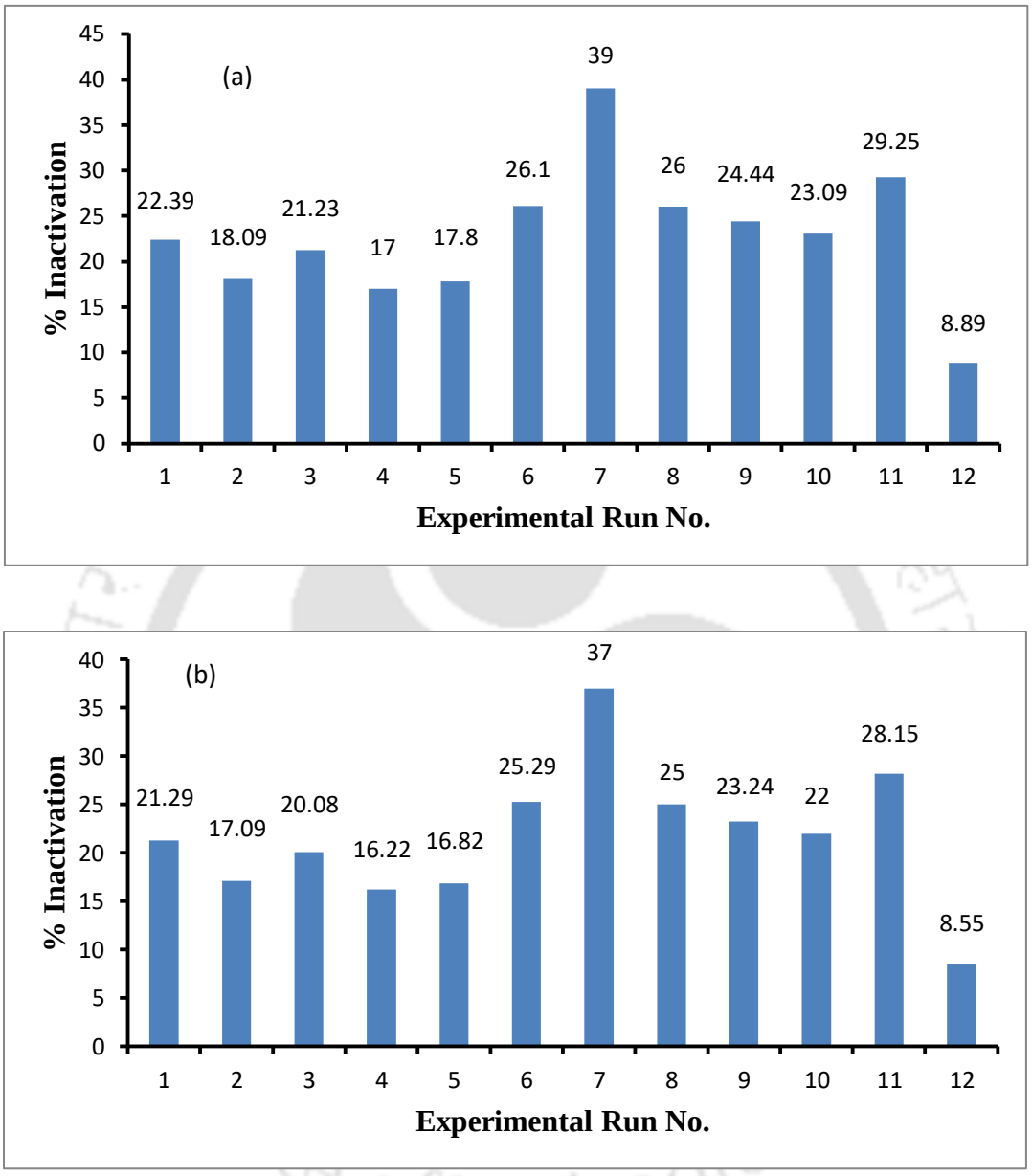


Fig. 4.19 Photo-inactivation obtained in the different experimental runs using SAQS for 30 minutes dark incubation period (a) *E. hirae* (b) *E. coli*

The expectation was to achieve 48% inactivation for *E. hirae* and 46% for *E. coli* using MB whereas 39% and 37 % inactivation for *E. hirae* and *E. coli* respectively using SAQS in all the runs, as the process parameters chosen has shown such results in aqueous solution. The results in

this experiment showed maximum photo-inactivation only for run no. 7 where any organic and inorganic components were not present, as compared to the other experimental run's of Plackett Burman design (table 3.8). Hence, the reason for decrease in the photoinactivation efficiency could be attributed to the presence of organic and inorganic components in the MSWW. The inhibition of photoinactivation in the presence of these components could be explained by effects such as radical scavenging, absorption of light by the organic components or competition between organic components and bacteria for reactive oxygen species formed (Gomes et al., 2009; Rincon and Pulgarin, 2004).

Methylene blue is known to follow Type I mechanism for the production of reactive oxygen species, which forms hydroxyl radical ($^{\bullet}OH$) (Sabbahi et al., 2008) whereas sodium anthraquinone-2- sulphonate is known to produce singlet oxygen as well as hydroxyl radical (Liu and Sun, 2011).

Hydroxyl radicals are the most powerful oxidants and can easily attack organic molecules leading to the production of organic peroxide radicals which finally gets converted to CO_2, H_2O and inorganic salts (equation 1-3) (Antoniadis et al., 2007).

$$RH + {}^{\bullet}OH \longrightarrow R^{\bullet} + H_2O \quad (1)$$

$$R^{\bullet} + O_2 \longrightarrow RO_2^{\bullet} \quad (2)$$

$$R \longrightarrow RO_2^{\bullet} \longrightarrow CO_2 + \text{inorganic salts} \quad (3)$$

Availability of iron in the wastewater can also produce hydroxyl radical by photo-fenton reagent (Antoniadis et al., 2007) but the radical formed is scavenged by organic molecules present instead of bacterial inactivation (equation 4-6).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-} \quad (4)$$

$$Fe^{3+} + H_2O + h\nu \longrightarrow Fe^{2+} + {}^{\bullet}OH + H^{+} \quad (5)$$

$$R\text{-}H + {}^{\bullet}OH \longrightarrow ROO^{\bullet} \longrightarrow CO_2 + \text{inorganic salts} \quad (6)$$

Rincon and Pulgarin (2004) reported inhibition of bacterial inactivation in the presence of monovalent cations like Na^+ or K^+ and quenching of the hydroxyl radical due to the presence of anions like HCO_3^-, HPO_4^{2-}, $H_2PO_4^-$, SO_4^{2-} and Cl^-. Hence, we could conclude that even though reactive oxygen species are being produced by mb, SAQS and Fenton reagent still decrease in bacterial inactivation is observed, which could be due to the quenching of ROS by organic components.

The results have revealed minimum inactivation in the case of *E. coli* using SAQS and maximum inactivation for *E. hirae* using MB. This phenomenon could be explained, as more MSWW components show significant effect on *E. coli* inactivation using SAQS as compared to *E. hirae* using MB, by scavenging ROS formed during photoinactivation. Also the presence of wastewater components makes the solution turbid compare to pure distilled water with PBS, which hinders light penetration in the solution and reduces the photoinactivation efficiency. The low inactivation efficiency in case of *E. coli* can also be attributed to the presence of lipopolysaccharide membrane which prevents direct interaction of photosensitizers with cell membrane.

4.5.2 Statistical analysis

To better understand the role of different MSWW components on the inactivation of *E. coli* and *E. hirae* in the presence of MB and SAQS, statistical analysis of the results in the form of ANOVA and student't' test was performed. The ANOVA (Tables 4.13 and 4.14) for photo-inactivation results of *E. hirae* and *E. coli* using MB and SAQS obtained at 30 minutes dark incubation period, with the high Fischer's 'F' value and a low probability 'P' value of the regression model indicates its validity in explaining the variations in the results. Accuracy and precision of the models, in the form of determination coefficient (R^2), adjusted R^2, standard deviation (SD) and predicted residual error sum of squares (PRESS), suggest that the models were highly efficient in predicting the experimental photo-inactivation results (Mahanty et al., 2010).

Table 4.13 ANOVA for effect of synthetic wastewater components on viable cell count of *E. hirae* at the end of inactivation at 30 minutes dark incubation period

	***E. hirae* + SAQS**						***E. hirae* + MB**					
	F	P	R^2	R^2 Adj	SD	PRESS	F	P	R^2	R^2 Adj	SD	PRESS
Main effects	12.72	0.00	92.1	84.8	17.9	15538	2.11	0.10	65.9	34.7	21.0	21224
Urea	36.29	0.00					4.90	0.04				
NH_4Cl	0.33	0.57					1.27	0.28				
CH_3COONa	5.62	0.03					3.47	0.08				
Yeast	0.12	0.73					0.03	0.86				
Peptone	12.29	0.00					0.54	0.47				
Starch	34.14	0.00					3.77	0.07				
KH_2PO_4	2.01	0.18					0.01	0.93				
$FeSO_4$	0.03	0.86					0.05	0.82				
$CuCl_2$	6.52	0.02					1.06	0.32				
$MnSO_4$	2.08	0.17					3.47	0.08				
Milk Powder	40.51	0.00					4.64	0.05				

Table 4.14 ANOVA for effect of synthetic wastewater components on viable cell count of *E. coli* at the end of inactivation at 30 minutes dark incubation period

	***E. coli* +MB**						***E. coli* + SAQS**					
	F	P	R^2	R^2 Adj	SD	PRESS	F	P	R^2	R^2 Adj	SD	PRESS
Main effects	3.91	0.013	78.2	58.2	16.5	13090	16.87	0.00	93.9	88.36	22.8	25108
Urea	5.93	0.031					47.47	0.00				
NH_4Cl	0.03	0.875					3.86	0.07				
CH_3COONa	0.81	0.385					1.70	0.21				
Yeast	0.00	0.952					5.14	0.04				
Peptone	0.50	0.495					16.27	0.00				
Starch	0.06	0.818					4.66	0.05				
KH_2PO_4	3.48	0.087					5.81	0.03				
$FeSO_4$	1.38	0.263					17.60	0.00				
$CuCl_2$	0.00	0.971					45.53	0.00				
$MnSO_4$	2.46	0.142					16.27	0.00				
Milk Powder	28.38	0.000					21.21	0.00				

It can be clearly stated urea, sodium acetate, peptone, starch, copper chloride and milk has adversely affected the photoinactivation of *E. hirae* using SAQS, whereas in case of E. hirae inactivation using MB only urea and milk has some negative effect on the process (table 4.13). In case of *E. coli* except ammonium chloride and sodium acetate all the other wastewater component showed negative impact on photoinactivation using SAQS, but when MB was used as photosensitizer except milk no other pollutant shows any significant impact on the process (table 4.14). The estimated coefficients of individual effects of the MSWW components presented in Tables 4.15 and 4.16, as well confirmed these results. These tables revealed significant effect of various MSWW components on bacterial inactivation by MB or SAQS depending on the combination of bacterial strain and the photosensitizer. In case of *E. hirae* inactivation using SAQS Table 4.15 revealed significant effect of urea, sodium acetate, peptone, starch, copper chloride and milk powder whereas only urea showed significant effect when MB

is used. Results for *E. coli* inactivation using SAQS (Table 4.16) revealed significant effect of urea, yeast, peptone, potassium dihydrogen phosphate, ferrous sulphate, copper chloride, manganese sulphate and milk powder whereas using MB, urea, potassium dihydrogen phosphate and milk powder showed significant effect.

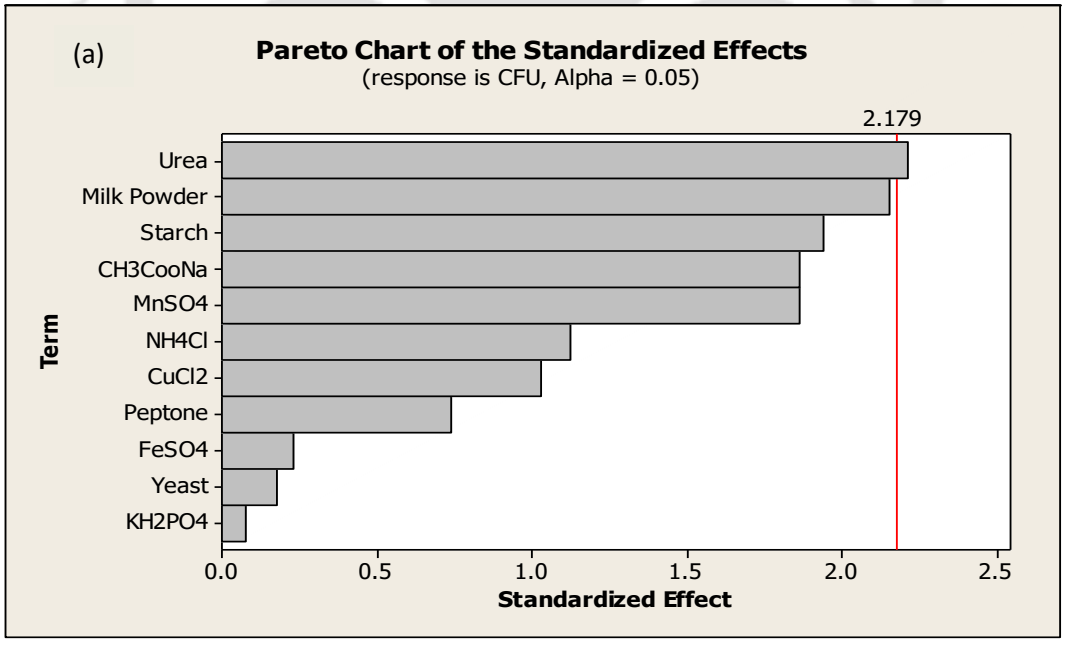
All the results for effect of synthetic wastewater components on the photo-inactivation of *E.coli* and *E. hirae* are depicted in a better way in the form of pareto charts and are illustrated in Fig. 4.20 and 4.21. Horizontal bars in these charts represent effects (i.e. individual terms) of the parameters and the effects which extend past the reference line (vertical line on the chart) denote the significant ones ($\alpha = 0.05$).

Table 4.15 Student't' test of the regression coefficients for synthetic wastewater components for *E. hirae*

	***E. hirae* + SAQS**			***E. hirae* + MB**		
Term	**Coeff.**	**T**	**P**	**Coeff.**	**T**	**P**
Constant	344.58	93.79	0.00	326.08	75.97	0.00
Urea	22.125	6.02	0.00	9.50	2.21	0.04
NH_4Cl	2.125	0.58	0.57	4.83	1.13	0.28
CH_3COONa	-8.708	-2.37	0.03	8.00	1.86	0.08
Yeast	1.292	0.35	0.73	0.75	0.17	0.86
Peptone	12.875	3.51	0.00	3.16	0.74	0.45
Starch	21.458	5.84	0.00	8.33	1.94	0.07
KH_2PO_4	5.208	1.42	0.18	0.33	0.08	0.93
$FeSO_4$	-0.625	-0.17	0.86	1.00	0.23	0.82
$CuCl_2$	-9.375	-2.55	0.02	4.41	1.03	0.32
$MnSO_4$	-5.292	-1.44	0.17	-8.00	-1.86	0.08
Milk Powder	23.375	6.36	0.00	9.25	2.16	0.05

Table 4.16 Student't' test of the regression coefficients for synthetic wastewater components for *E. coli*

	***E. coli* + MB**			***E. coli* + SAQS**		
Term	**Coeff.**	**T**	**P**	**Coeff.**	**T**	**P**
Constant	293.12	86.96	0.00	301.92	64.67	0.00
Urea	8.20	2.44	0.03	-32.17	-6.89	0.00
NH_4Cl	0.54	0.16	0.87	9.17	1.96	0.07
CH_3COONa	3.04	0.90	0.38	6.08	1.30	0.21
Yeast	0.20	-0.06	0.95	10.58	2.27	0.04
Peptone	2.37	0.70	0.49	-18.83	-4.03	0.00
Starch	-0.79	-0.23	0.81	-10.08	-2.16	0.05
KH_2PO_4	6.29	1.87	0.08	11.25	2.41	0.03
$FeSO_4$	-3.95	-1.17	0.26	19.58	4.19	0.00
$CuCl_2$	-0.12	-0.04	0.97	31.50	6.75	0.00
$MnSO_4$	5.29	1.57	0.14	18.83	4.03	0.00
Milk Powder	17.95	5.33	0.00	21.50	4.61	0.00



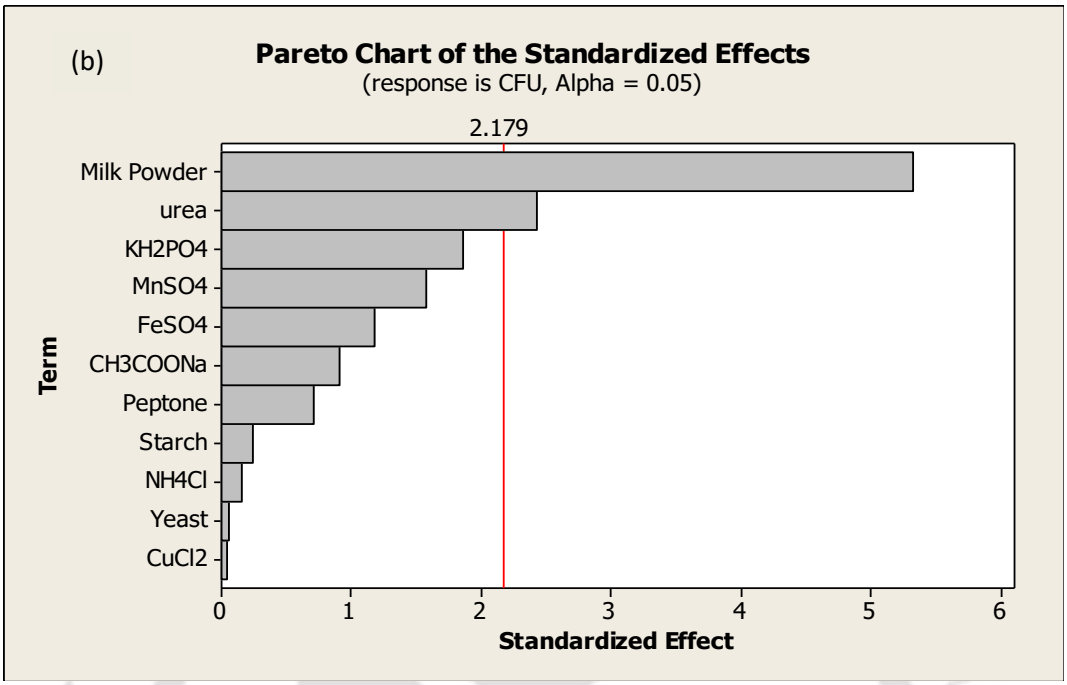


Fig. 4.20 Pareto chart showing the effect of different variables on photo-inactivation using MB (a) *E. hirae* and (b) *E. coli*

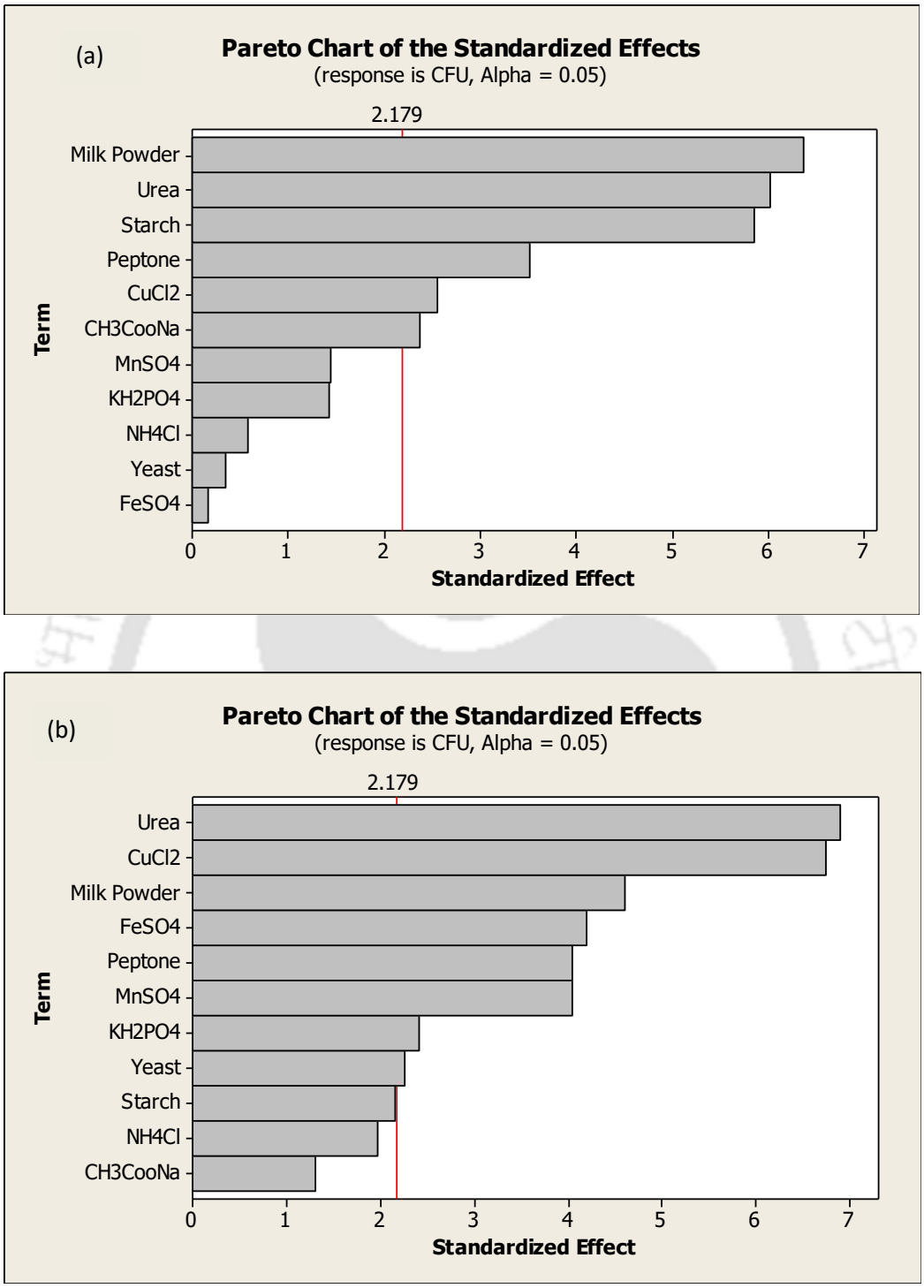


Fig. 4.21 Pareto chart showing the effect of different variables on photo-inactivation using SAQS (a) *E. hirae* and (b) *E. coli*

CHAPTER 5
SUMMARY AND CONCLUSION

The present research work investigated photo-inactivation of indicator microorganisms *Escherichia coli* and *Enterococcus hirae* in aqueous solution using photoactive compounds methylene blue (MB) and sodium anthraquinone-2- sulphonate (SAQS). It also investigated the effect of synthetic wastewater components on inactivation efficiency of these photosensitive compounds.

The results of photo-inactivation obtained in the study involving individual photoactive dyes revealed that both cationic photosensitizers are efficient against Gram positive and Gram negative bacterial strains, with a higher efficiency against the Gram positive *E. hirae.* Gram positive bacterial strains are found to be more susceptible to inactivation due to the absence of lipopolysaccharide membrane. Between the two PS, MB was more efficient against both the bacterial strains than SAQS at the same concentration, pH of the solution and viable cell count values. Cell cytometric analysis further revealed that the mechanism of photo-inactivation involved bacterial cell membrane damage by the PS. This is reported in the form of shift in the fluorescence peak of the propidium iodide with increasing concentration of the PS. Propidium iodide is known to bind with the DNA of the cells whose membrane is compromised. The effect of UV obtained was very high (99% efficiency) in contrast to the photosensitizers employed which yielded a maximum efficiency of 48.55%. Considering the cost of UV, besides its harmful effect to human upon exposure and difficulty in implementation of UV radiation for disinfection (Metcalf and Eddy, 2003), dye sensitized photoinactivation of bacterial strains holds a great promise for the future. Statistical analysis of the results revealed that besides the significant individual effect due to concentration of PS, pH of bacterial

suspension and dilution, interaction effect between concentration of PS and initial viable cell count was significant for the bacterial inactivation. Further, the results of lipid peroxidation and protein carbonyl assay revealed the change in membrane protein and lipids as compared to the control. High lipid peroxidation and protein carbonylation levels were observed for *E. hirae*as compared to *E. coli* when treated with MB and SAQS. MB also showed higher assay values as compared to SAQS. These results are found to be in accordance with the results obtained by colony counting method.

Compared to these results, when MB and SAQS were added together to the same volume of bacterial suspensions of either *E. hirae* or *E. coli* an increase in the percent inactivation for both the strains was observed. The inactivation efficiency increased by 1.5 to 2 % as compared with inactivation efficiency of individual dyes in suspension. Statistical analysis of the results revealed that MB has significant effect in the case of Gram positive *E. hirae* whereas the effect is insignificant for gram negative *E. coli* (due to the presence of external lipopolysaccharide coat). It also revealed that other individual and interaction effect were insignificant.

Further, the results for the effect of synthetic wastewater components on photo-inactivation showed a reduction in photo-inactivation efficiency. When more components of the synthetic wastewater have significant effect on inactivation then a higher degree of reduction in inactivation is observed as compared to when fewer components are showing significant effect. In case of *E. coli* and SAQS the observed inactivation efficiency is minimum whereas *E. hirae* treated with MB shows maximum inactivation efficiency because only one component “urea”is found to have significant effect. This reduction in inactivation efficiency can be attributed to the shielding effect of organics on bacterial inactivation due to radical scavenging, inhibition of the PS by reacting with wastewater components and absorption of light.

Overall, the present study showed that the cationic dyes are efficient for both Gram +ve and Gram –ve bacterial inactivation in aqueous solution with more efficiency for Gram +ve bacteria due to the absence of lipopolysachharide membrane. Enhancement in the photoinactivation efficiency was observed in the presence of more than one dye but the presence of organic and inorganic components in the aqueous solution was found to protect microorganisms from photoinactivation.

Scope for Future Work

The present research work focused on photo-inactivation of model organisms *Escherichia coli* and *Enterococcus hirae*using methylene blue and sodium anthraquinone-2- sulphonate in aqueous solution and synthetic municipal wastewater. The following are suggested as future work to continue in this area of research

1) Performance evaluation of the photoactive dyes using bioreactors under different operating conditions like wastewater hydraulic retention time, light exposure, initial microbial counts etc.
2) Photo-inactivation of microorganisms in real waste water from sewage, slaughter house or restaurants.
3) Studying the mechanism of photoinactivation by studying the exact damage to microbes i.e cell disruption, DNA damage or inactivation of any pathway or enzyme system.
4) Improving the photoinactivation efficiency by testing novel dyes or encapsulating the dyes in gel beads or capsules.
5) Testing the efficiency against pathogenic microorganisms.

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LIST OF PUBLICATIONS

Journal Publications

1. Singh, M., Pakshirajan, K. and Trivedi, V. (2016) Photo-inactivation of *Escherichia coli* and *Enterococcus hirae* using methylene blue and sodium anthraquinone-2-sulphonate: effect of process parameters. 3 Biotech. In press. DOI: 10.1007/s13205-016-0487-6.
2. Singh, M., Pakshirajan, K. and Trivedi, V. (2016) A Study on combined effect of Methylene blue and Sodium anthraquinone-2- sulphonate on inactivation efficiency of *Escherichia coli* and *Enterococcus hirae*. International Journal of Chemtech. International Journal of ChemTech Research, 2016, 9(6), pp 614-619.
3. Singh, M., Pakshirajan, K. and Trivedi, V. (2016) Photo-inactivation of *Escherichia coli* and *Enterococcus hirae* using methylene blue and sodium anthraquinone-2-sulphonate in synthetic wastewater. (Manuscript under preparation).
4. Singh, M., Pakshirajan, K. and Trivedi, V. (2016) An overview of Photo-inactivation technology as advanced wastewater disinfection methodology. (Manuscript under preparation).

Conference proceedings

1. Singh, M., Pakshirajan, K. and Trivedi, V. Reactive oxygen species mediated inactivation of microorganisms in wastewater. Abstract published in international conference on industrial biotechnology (ICIB) 2012.
2. Singh, M., Pakshirajan, K. and Trivedi, V. Photo-inactivation of *Escherichia coli* and *Enterococcus hirae* using methylene blue and sodium anthraquinone-2-sulphonate: effect of process parameters. Abstract published in international conference on advances in biotechnology and bioinformatics (ICABB) 2013.