

Photocatalytic Degradation of *E. coli* and *S. aureus* by Multi Walled Carbon Nanotubes

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Abstract

Carbon Nano Tubes could be either metallic or semi-conducting in nature, depending on their diameter. Its photocatalytic behavior has given an impetus to use it as an anti-microbial agent. More than 95% *Escherichia coli* and *Staphylococcus aureus* bacteria got killed when exposed to Carbon Nano Tubes for 30 minutes in presence of sunlight. Carbon Nano Tubes are supposed to have smooth surface on to which it accumulates positive charges when exposed to light. The surface that is non illuminated has negative charge. At the cellular level microorganisms produce negative charges on the cell membrane, Therefore damaging effect of multi walled carbon nano tubes (exposed to light) on the microorganisms is possible. In this paper, photo catalytic killing of microbes by multi walled carbon nano tubes is reported. Killing was due to damage in the cell membrane, as seen in SEM micrographs. Moreover biochemical analysis of membrane as well as total cellular proteins by SDS PAGE showed that there was denaturation of membrane proteins as well as total proteins of both the microbes studied. The killed microbes that showed a decrease in number of protein bands (i.e. due to breaking down of proteins) also showed an increase in level of free amino acids in microbes. This further confirmed that proteins got denatured or broken down into shorter units of amino acids. Increased level of free amino acids was recorded in both the microbes treated with multi walled carbon nano tubes and sunlight.

Keywords : Photocatalysis, Multi walled Carbon nanotubes, *Escherichia coli*, *Staphylococcus aureus*

1. Introduction

Discovery of fullerenes provided exciting insights into carbon nanostructures. The specific architectures of Carbon Nano Materials (CNM) built from sp² carbon units based on simple geometrical principles have been found to result in new symmetries and structures [1] that have fascinating and useful properties.

Nearly a decade after their discovery, biologists covet the uses of Carbon Nano Tubes (CNTs) for various applications, because of the scale and their unique and novel structural, mechanical, electronic, thermal and chemical properties.

Sharon *et al.*, 2005 [2] have postulated that when a semiconductor (either CNT or iron oxide) is in contact with any other materials like solution of some inorganic salt or a buffer solution, a depletion region is formed like one forms a metal-Schottky junction at the interface of CNT and inorganic salt solution. When this depletion region is illuminated with light source of energy equivalent to the band gap of the material, the interface gets accumulated with photogenerated electrons and holes. These photogenerated carriers are highly

reactive and when they come in contact with any organic material, the latter is completely decomposed by oxidation/reduction process. Since all living organisms are made up of macromolecules like proteins, such photo catalytic action will also decompose the cell wall proteins leading to destruction of the cell wall [2]. Killing microbes is commonly achieved by certain chemical agents which often adds to the environmental pollution due to toxicity. Two inorganic approaches can be used for killing microbes either by the photocatalytic activity or by exploiting the toxicity of certain metallic cations such as silver [3, 4]. Photocatalytic killing of microbes by CNT has already been shown [2 and the references there in]. It was suggested by Sharon *et al.* that carbon nanomaterials of specific band gap (band gap 1.2 eV) can possess the property of semi-conducting nature depending on its internal diameter (between 20-50 nm), that may damage the cell membrane due to photocatalytic activity of CNT causing death of microbes. Though Sharon *et al.* [2] have postulated this hypothesis, in the present work we have tried to give an experimental proof for the photo catalytic degradation of membrane proteins which causes the death of microbes.

2. Experimental

2.1. Selection of Microbes

Two microbes having different composition of cell membrane i.e. *Escherichia coli* NCIM 5051 (Gram negative coccobacilli) and *Staphylococcus aureus* NCIM 2079 (Gram positive cocci); procured from National Chemical Laboratories, Pune, India were selected for the present work.

Cultures were maintained on 'Sterile Nutrient Agar' at pH 7.2 for growth. Growth curve of microbial cultures were found out by following the increase in cell mass at regular time interval and by measuring turbidity at 520 nm with a spectrophotometer. Cultures in the mid log phase of growth were taken for the experiment.

During exposure of microbes to Multi Walled Carbon Nanotubes (MWCNT) and sunlight they were grown on 'Sterile Nutrient Broth'. MWCNT was procured from MONAD Nanotech Pvt. Ltd. India, having following specifications: Synthesized by: CVD process from camphor, 20-40 nm external diameter and 5-15 μm length, MWCNT content 95% having 40-300 m^2/g specific surface area and 1812 ± 300 W/m-K thermal conductivity, 2% amorphous Carbon and $\leq 2\%$ Ash. MWCNTs were further purified by acid (HCl) treatment to remove the metal catalyst, amorphous carbon and ash present in the MWCNT sample.

2.2. Treatment of *Escherichia coli* and *Staphylococcus aureus* with MWCNT and exposure to sunlight

Loopful of 12 h old cultures (in mid log phase) of *Escherichia coli* and *Staphylococcus aureus* were taken in 100 ml 'Sterile Nutrient Broth' having required concentrations (0, 10, 100 & 500 mg/l) of MWCNT. Six replica of each treatment was prepared in flasks.

25 ml of culture from each of the above flasks were aseptically transferred on sterile petriplates and were exposed to dark and sunlight for 30 min. The experiments were performed at 10 am when the intensity of sunlight was 20,000 lux.

Effect of MWCNT and sunlight was studied by recording the survival of microbes, scanning electron microscopy, membrane and total protein analysis by electrophoresis and total protein and free amino acid content by biochemical assay.

2.3. Mortality of microbes treated with MWCNT and sunlight

After exposure to the sunlight and MWCNT, viability of both the microbes were checked by spread plate method using Sterile Nutrient Agar. Each bacterium would thus result in production of one colony, which was counted to determine the number of surviving bacteria in the given

suspension. Dense suspensions of cells were suitably diluted to avoid over-crowding of the colonies on the medium. A serial dilution from 10^{-1} to 10^{-5} using 4.5 ml of sterile saline and 0.5 ml of respective culture was prepared. Aliquot of 0.1 ml of respective dilution was spread on to the Sterile Nutrient Agar plate with the help of a glass spreader. Plates were inverted and incubated at 37°C for 24 h. Number of colonies developed on the plate were counted to calculate the viability of the microbes. Thus the mortality due to treatment was assessed. Following assays of both the microbes were done, after they were exposed to MWCNT and sunlight.

2.4. Membrane and total protein assay (by SDS-PAGE methods) of microbes treated with MWCNT and sunlight [5]

2.4.1. Extraction of membrane protein

Treated and control cultures of *E. coli* and *S. aureus* were spun in the medium at 6000 rpm and 2°C for 10 min. Pellets formed were re-suspended in 25 ml of 50 mM Tris HCl pH 8.5 and 2 mM EDTA and sonicated for 30 sec, four times, with 20 sec gap for cooling. The tube with cells was kept in crushed ice.

Supernatant was again spun in Beckmann centrifuge at 25,000 g (2°C) for 10 min. Pellet formed were of membrane protein; they were re-suspended in 2 mM Tris-HCl (pH 7.8); and stored in a screw-capped vial in the deep freeze (-20°C).

2.4.2. Extraction of Total Protein

1 ml of treated and control cultures of *E. coli* and *S. aureus* was added to 5 ml of phosphate buffer and then centrifuged at 8000 rpm for 20 mins. Supernatant was collected and extraction step was repeated 4 times. The supernatants were pooled together and the volume was made to 50 ml with phosphate buffer.

To 1 ml of the above extract, 1 ml 20% Tri Chloro-Acetic acid was added and after half an hour centrifuged at 8000 rpm for 20 min. Pellet was washed twice with acetone and again centrifuged. Supernatant was discarded and the pellet was mixed in 5 ml of 0.1 N NaOH.

2.4.3. Gel for SDS PAGE

5% of Stacking gel and 10% of Resolving gel were used. SDS-PAGE was carried out using membrane and total protein samples.

2.4.4. Protein Sample Preparation for Electrophoresis

The sample protein was denatured in 2 ml gel sample buffer containing 1.25 ml 0.5 M Tris-HCl (pH 6.8), 1 ml β -mercapto-ethanol, 2 ml glycerol; 0.4 ml 1% bromophenol blue to 0.4 g SDS and the final volume was made to 10 ml with distilled water.

25 μl of the above mentioned protein samples were loaded

in each well of the electrophoretic gel. GENE I Protein Molecular Weight Marker™ RPMW-H, having a range of 29-205 kDa, mol. wt. was used as standard protein.

Initially 10-15 mA current was applied for 10-15 min i.e. until the samples started traveling through the stacking gel. Then current supply was increased to 30 mA until the bromophenol blue dye reached near the bottom of the gel slab. The gel slab was kept in a trough containing staining solution (200 ml methanol + 35 ml glacial acetic acid + 1.25 g Coomassie Brilliant Blue R-250 made to 500 ml by adding distilled water) until clear bands were observed. Excess stain was removed by keeping the gel in de-staining solution (75 ml of glacial acetic acid + 50 ml of methanol final volume made to 1 liter. with distilled water).

2.5. Biochemical assay of total cellular protein [6], of microbes treated with MWCNT and light

2.5.1. Protein Estimation

To 1 ml aliquot of the treated and untreated microbial suspension, 5 ml of freshly prepared alkaline copper sulphate reagent was added; after 10 min 0.5 ml of Folin's reagent was added, mixed well and allowed the color to develop for 30 minutes. Absorbance was recorded at 660 nm after setting the instrument with reagent blank which contains 1ml 0.1 N NaOH instead of sample aliquot. Standard curve with graded concentration of Bovine Serum Albumin was plotted.

2.6. Free amino acid assay by Ninhydrin method [5]

2.6.1. Free amino acids extraction

was done by adding 70% ethanol to the microbial suspension. Homogenate was centrifuged at 5000 rpm for 10 min. Supernatant was decanted. This step was repeated 4 times. All supernatants were pooled together and final volume was made to 50 ml with 70% ethanol.

2.6.2. Sample preparation

10 ml of ethanol extract was evaporated to dryness on a boiling water bath and the residue was dissolved in 5 ml of 0.2 M citrate buffer (pH 5.5).

2.6.3. Free amino acid estimation

To 2 ml of the above sample, 1 ml of Ninhydrin reagent was added and kept in boiling water bath for 20 min. It was cooled and volume was made to 10 ml with distilled water. Absorbance was recorded at 570 nm using 0.2 M citrate buffer pH 5.5 as blank. Simultaneously a standard curve using graded concentrations of glycine (0-100 µg) was plotted.

2.7. Morphological Characterization, of *E. coli* and *S. aureus* treated with MWCNT and sunlight by SEM

Treated and control samples of microbes were dehydrated in acetone and the SEM micrographs were taken in a Co-Joel (SEM-3010).

3. Results and Discussion

3.1. Percentage Survival/Mortality of *E. coli* and *S. aureus* exposed to MWCNT and sunlight

Survival of *E. coli* and *S. aureus* cells after 30 minutes of exposure to different concentrations (10, 100, 500 mg/l) of MWCNT and sunlight was recorded by Colony Count (Table 1 and 2). It must be mentioned here that during growth cycle microbes do perish due to natural death, hence those microbes which were not exposed to MWCNT (Control) also exhibited some mortality. The surviving microbes in control were taken as 100% survival and the rest of the mortality/survival was calculated after deducting them.

Tables 1 and 2 very clearly give an indication of photocatalytic activity of MWCNT that caused the mortality of *E. coli* and *S. aureus*. When both the microbes were grown in sunlight with MWCNT (100, 500 mg/l) barely 1-3% micro-

Table 1. Percentage mortality/survival of *E. coli* exposed to different concentrations of MWCNT for 30 minutes in dark and sunlight

Treatment given to <i>E. coli</i> for 30 minutes	Colony Count [cfu/ml]	% survival [(x/394)×100] dilution used=10 ⁻³	% Mortality
Culture (control)	3.944×10 ⁵	100	0
Culture + Dark	3.94×10 ⁵	99.98	0.02
Culture + Dark + 10 ppm CNT	3.67×10 ⁵	93.05	6.95
Culture + Dark + 100 ppm CNT	3.546×10 ⁵	89.90	10.1
Culture + Dark + 500 ppm CNT	3.546×10 ⁵	89.90	10.1
Culture + Sunlight	2.784×10 ⁵	70.58	29.42
Culture + Sunlight + 10 ppm CNT	1.471×10 ⁵	37.29	62.71
Culture + Sunlight + 100 ppm CNT	0.127×10 ⁵	3.22	96.78
Culture + Sunlight + 500 ppm CNT	0.056×10 ⁵	1.41	98.53

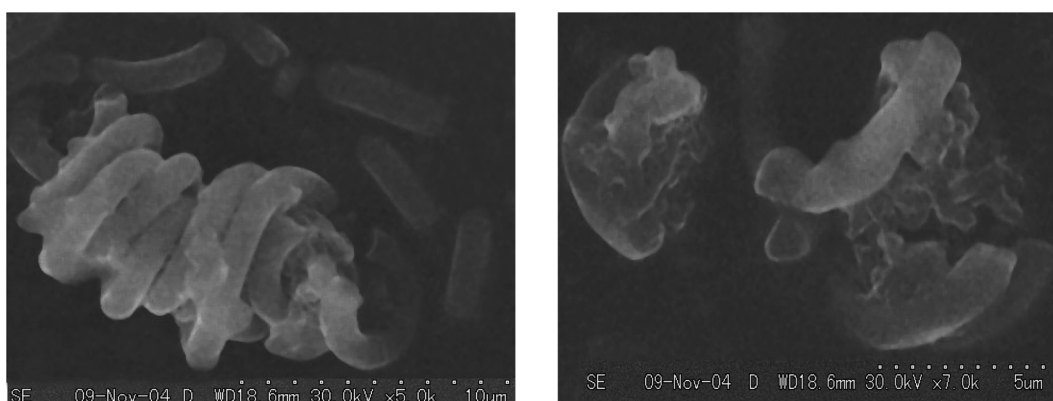
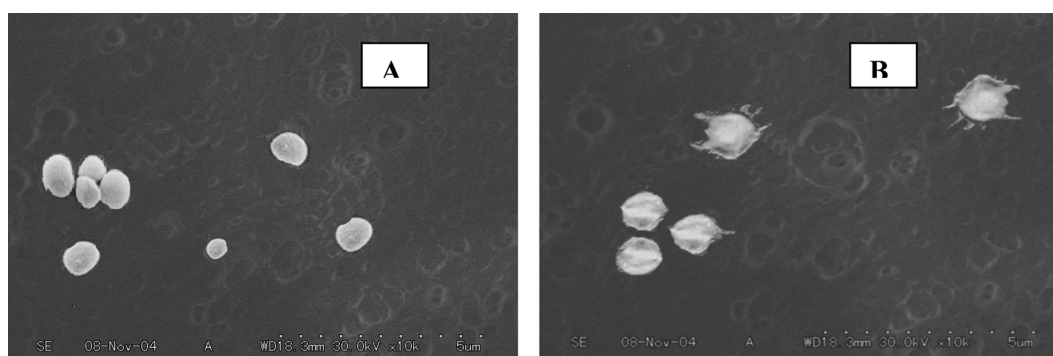
Table 2. Percentage survival/mortality of *S. aureus* exposed to different concentrations of MWCNT for 30 minutes I dark and sunlight

Treatment given to <i>S.aureus</i> for 30 minutes	Colony Count per ml (dilution used: 10^{-3}) [cfu/ml]	% survival $[(x/394)\times 100]$ dilution used= 10^{-3}	% Mortality
Culture (control)	4.571×10^5	100	0
Culture + Dark	4.562×10^5	99.80	0.2
Culture + Dark + 10 ppm CNT	4.464×10^5	97.65	2.35
Culture + Dark + 100 ppm CNT	4.427×10^5	96.84	3.16
Culture + Dark + 500 ppm CNT	4.414×10^5	96.56	3.44
Culture + Sunlight	3.760×10^5	82.25	17.75
Culture + Sunlight + 10 ppm CNT	1.381×10^5	30.21	69.79
Culture + Sunlight + 100 ppm CNT	0.043×10^5	0.94	99.06
Culture + Sunlight + 500 ppm CNT	0.068×10^5	1.48	98.52

bes could survive i.e. indicating a mortality of more than 98%. It was interesting to note that photocatalytic damaging effect of MWCNT and sunlight was almost the same for both *E. coli* and *S. aureus* (Table 1, 2) i.e. within 30 min of exposure, maximum mortality occurred. So far as the impact of concentration of MWCNT with which microbes were treated i.e. 10, 100, 500 mg/l, is concerned all the three

concentrations were fatal. With the increase in concentration, the mortality increased for both the tested microbes.

The SEM micrographs presented in the Fig. 1 and 2 show disintegrated cells and ruptured cell membranes of the microbes treated with MWCNT and visible light. Hence it can be concluded that the mortality was due to the rupture of cell membrane of microbes.

**Fig. 1.** Scanning Electron Micrographs of *E. coli* treated with 100 ppm of MWCNT and Sunlight; showing ruptured cell membranes as well as some disintegrated cells of *E. coli*.**Fig. 2.** Scanning Electron Micrographs of *S. aureus* treated with 100 ppm of MWCNT and Sunlight showing (A) healthy cells and (B) cells with ruptured cell membrane.

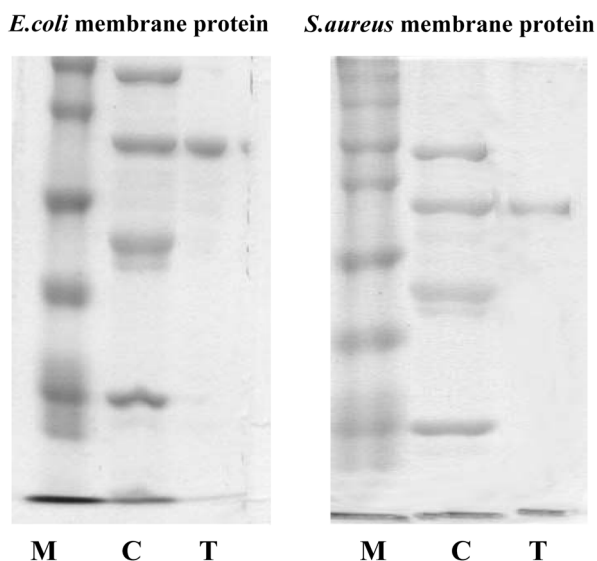


Fig. 3. SDS-PAGE of membrane proteins of *E. coli* and *S. aureus* treated with 100 mg/l MWCNT in dark (C), light (T) and Marker Protein (M). (T of both the microbes is showing only one band of membrane protein a compared to five bands of membrane protein of control, after denaturation due to photocatalytic activity of MWCNT).

3.2. Membrane and Cellular protein

It was decided to study whether membrane rupture as can be seen in Fig. 1 and 2 was due to photocatalytic degradation of membrane and total cellular proteins or not.

Gel Electrophoresis analysis of membrane protein very clearly (Fig. 3) shows that when microbes were treated with MWCNT and light, only one clear protein band was observed in both the microbes. Whereas untreated *E. coli* had 6 and *S. aureus* had 8 protein bands.

Absence of 2 protein bands in SDS PAGE of *E. coli* and 3 in *S. aureus* was also noted when total cellular protein was analyzed. Thus confirming the theory that MWCNTs can photocatalytically degrade the proteins (Fig. 4).

3.3. Biochemical analysis of total protein

Table 3 also indicated reduction in protein content when

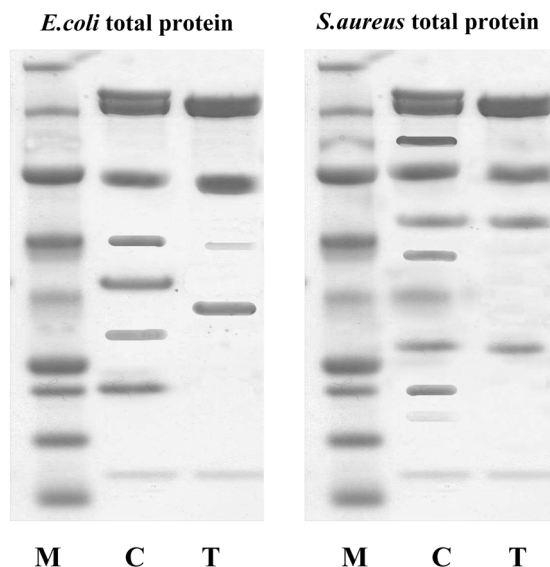


Fig. 4. SDS-PAGE of Total cellular proteins of *E. coli* and *S. aureus* treated with 100 mg/l MWCNT in dark (C) and light (T) and Marker Protein (M). (T of *E. coli* is showing only four bands of proteins as compared to eight bands in control and T of *S. aureus* is showing only four clear bands of proteins as compared to ten bands in control; after denaturation due to photocatalytic activity of MWCNT).

microbes were treated with MWCNT and light. The reduction in total protein content was more in *S. aureus* i.e. approximately 80%, where as it was about 70% in *E. coli*. The percentage is as compared to initial protein content. May be that is why only 1% *S. aureus* could survive (i.e. 99% mortality) when exposed to 500 mg/l MWCNT. Survival of *E. coli* 2% for the same treatment. Suggesting that microbial death is due to photocatalytic degradation of membrane as well as total cellular protein.

3.4. Free amino acids

There are two major types of bacteria: Gram-positive and Gram-negative. The cell wall of *Gram-positive bacteria* consists of many polymer layers of Peptidoglycan connected by amino acid bridges. The Peptidoglycan polymer is composed of an alternating sequence of N-acetylglucosamine

Table 3. Total protein content in *E. coli* and *S. aureus* cells before and after the treatment with different concentrations of MWCNT and sunlight (20 K Lux)

SAMPLE	EFFECT OF CNT ON TOTAL PROTEIN CONTENT ($\mu\text{g/ml}$) IN MICROBES TREATED FOR 30 MINUTES			
	Control	10 mg/l CNT	100 mg/l CNT	500 mg/l CNT
<i>E. coli</i> + Dark	233 $\mu\text{g/ml}$	231 $\mu\text{g/ml}$	230 $\mu\text{g/ml}$	229 $\mu\text{g/ml}$
<i>E. coli</i> + Sunlight	235 $\mu\text{g/ml}$	150 $\mu\text{g/ml}$	87 $\mu\text{g/ml}$	72 $\mu\text{g/ml}$
<i>S. aureus</i> + Dark	283 $\mu\text{g/ml}$	280 $\mu\text{g/ml}$	278 $\mu\text{g/ml}$	277 $\mu\text{g/ml}$
<i>S. aureus</i> + Sunlight	285 $\mu\text{g/ml}$	139 $\mu\text{g/ml}$	83 $\mu\text{g/ml}$	58 $\mu\text{g/ml}$

Table 4. Free Amino Acids content in *E. coli* and *S. Aureus* cells with and without the treatment with different concentrations of MWCNT and sunlight

SAMPLE	EFFECT OF CNT ON FREE AMINO ACIDS ($\mu\text{g/ml}$) IN MICROBES			
	Control	10 ppm CNT	100 ppm CNT	500 ppm CNT
<i>E. coli</i> + Dark	19	43	43	47
<i>E. coli</i> + Sunlight	16	102	132	151
<i>S. aureus</i> + Dark	42	45	48	57
<i>S. aureus</i> + Sunlight	40	122	131	148

and N-acetyl-muramic acid. Each Peptidoglycan layer is connected, or cross-linked, to the other by a bridge made of amino acids and amino acid derivatives. The cell wall of Gram-negative bacteria is much thinner, being comprised of only 20% Peptidoglycan.

An increased level of free amino acids (Table 4) in the treated microbes may be from the damaged wall structure i.e. freeing the amino acids from 'amino acid bridge' of peptide glycan or it may be due to breaking down of protein molecules of membrane or cellular protein.

In vitro action of carboxy- fullerene has been demonstrated by Teo *et al.* [7]. Fullerene compounds have avid reactivity with free radicals and are regarded as 'radical sponges'. And has been shown to be effective in the treatment of both Gram-positive and gram-negative infections, although its mode of action is poorly understood. However, they also envisaged that destruction of membrane integrity must have been the cause of bacterial death.

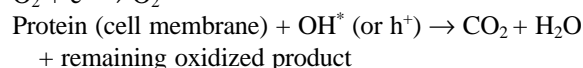
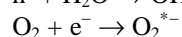
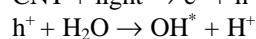
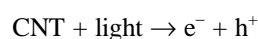
The above observation of mortality of microbes grown in presence of MWCNT and light confirm that photocatalytic activity of MWCNT and confirms that any organic material or water, present near the interface of semiconducting particles (MWCNT), would get quickly oxidized [8]. The chances are that proteins having disulfide groups are likely to be the ones that might have got affected by photocatalytic activity of MWCNT.

4. Photocatalytic Process of killing bacteria

Whenever a semiconductor material (in present study, semiconducting carbon with band gap 1.2 eV) comes in contact with another material of Fermi energy different to semiconductor, a depletion region is formed. This depletion region on exposure to band gap light, electron from valence and of the semiconductor gets excited to conduction band creating one electron/hole pair per photon absorbed. These electron/hole pairs have natural tendency to annihilate, but due to formation of depletion region, instead of getting annihilated are forced to react with materials in contact with the semiconductor [9, 10]. Since water is in far excess, water

reacts with these electron/hole pairs producing hydroxyl radicals, which are also very reactive. These hydroxyl radicals or electron photo-generated electron/hole pairs tend to react with any other organic materials present in their vicinity, causing oxidation (due to interaction with holes) or reduction (due to interaction with electron) [11-14].

Reaction sequence is given here:



Thus when the membrane of the cell comes in contact with either hydroxyl radicals or photo-generated holes, its protein would either get completely oxidized to carbon dioxide or it may get partly denatured releasing amino acids due to break in peptide bonds in a protein [7-9].

In the present experiment, SDS PAGE analysis has shown that when bacterial cells were exposed to Sun and MWCNT, the number of protein bands became less as compared to control. Absence of protein bands prove that protein component of the cell membrane as well as cell both got denatured by photocatalytic activity.

5. Conclusion

In presence of sun-light, semiconducting carbon (MWCNT) with a band gap of 1.2 eV can kill *E. coli* and *S. aureus*. It is proposed that the killing is due to photocatalytic denaturation of membrane as well as cell protein.

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