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STUDIES ON THE ENHANCEMENT OF THE PHOTOCATALYTIC INHIBITION OF MICROBIAL FOULING BY ANODIZED TITANIUM SURFACES

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ABSTRACT

Titanium, an other wise perfect condenser tube material in seawater applications is challenged by the problem of severe biofouling. Anatase, one of the two commercially important crystalline forms of titanium dioxide possesses excellent photocatalytic activity. It has been shown in our earlier studies that anodization of titanium produces anatase type of TiO_2 capable of photocatalytic inhibition of microbial adhesion under near-UV light illumination. The present study investigates the influence of anodizing voltage and anodizing time on the photocatalytic inhibition of *Pseudomonas* sp., a frequent colonizer of natural biofilms formed on titanium surfaces. The effect of heat treatment of anodized surfaces on photocatalytic activity was also studied. The surface oxide was characterized using Glancing Incidence X-ray Diffraction(GIXRD) and Atomic Force Microscopy (AFM). Attempts have also been made to understand the mechanism underlying the photocatalytic inactivation of the bacterial cells on TiO_2 surfaces by studying their growth characteristics.

Key words: photocatalytic activity, calcination, bactericidal effect, adhesion, rutile

1. INTRODUCTION

Semiconductor photocatalysis offers convenient routes to the purification of air and water and the provision of self-maintaining-clean surfaces [1,2]TiO_2 has attracted great deal of attention as a photocatalyst due to its excellent photochemical properties, non-toxicity and low cost and is considered a multifunctional material. It is used as a pigment, photocatalyst, filler, coating, photoconductor, UV filter, etc. TiO_2 appears in three crystalline polymorphic phases, rutile, anatase and brookite. Anatase phase of titania is preferred in dye sensitized solar cells [3] and catalysis whereas rutile is mostly used in the area of dielectrics[4] and high temperature oxygen gas sensors[5] . Matsunaga et al. reported for the first time in 1985 the antibacterial effect of TiO_2 photocatalytic reactions[6]. Today, photocatalytic antibacterial tiles, antifogging glass and air cleaners are among the commercial applications of the photocatalytic activity of TiO_2 based on its self cleaning ability[7].

Anodic oxidation (anodizing) is a commonly used surface treatment, especially on aluminum alloys for structural application to improve the corrosion or wear resistance[8] The application of anodic oxidation to the surfaces of titanium and its alloys is more recent. Anodization of titanium at room temperature forms titanium dioxide on the surface, which is predominantly anatase. Since, microbial fouling of titanium surfaces is the major problem with respect to the use of titanium in the sea water-cooled condensers of power plants, the self sterilizing ability of anatase type of TiO_2 thin films yielded the idea of growing a thin film of the anatase on the

biofouling prone titanium surface to reduce the attachment of these organic living cells, using the above mentioned industrially feasible process of anodization. Earlier studies conducted in our laboratory have shown that anodization of titanium in orthophosphoric acid resulted in the significant reduction of microbial adhesion. [9].

The present work focuses on investigating the influence of anodizing voltage, anodizing time and heat treatment of the anodized surfaces on photocatalytic activity in order to identify the appropriate anodizing conditions for maximum photocatalytic activity and thereby maximum inhibition of microbial attachment.

2. MATERIALS AND METHODS

2.1 Specimen Preparation

Commercially pure titanium grade-2 coupons (3cm x 2cm) were pickled in an acid bath (nitric acid 400 g/L + hydrofluoric acid 40 g/L+ water) and then ultrasonically cleaned using soap solution to remove all traces of acid from the surface, washed in running water and finally rinsed in distilled water and air dried. Anodization was carried out at 25^0C in orthophosphoric acid (30 g/L) for 10 min, 1h, 24h and 48h. at three voltages namely 30V, 50V and 100V. The acid pickled coupons were used as control.

2.1.1 Heat treatment

The samples prepared as described above after anodization were calcined at 500^oC for 3h. The calcined samples were removed from the furnace after the specified period and air-cooled. The photocatalytic activity of these heat-treated samples was also studied.

2.2 Evaluation of Photocatalytic Activity

The photocatalytic activity of the titanium surfaces anodized at various voltages, ie 30V, 50V, 100V for 10min, 1h, 24h and 48h was evaluated by methylene blue (MB) degradation method[7] using acid pickled coupons as control surfaces. Titanium coupons anodized at 30V, 50V and 100V as well as acid pickled control coupons, each six in number, were immersed in separate Petridishes containing 25ml methylene blue (2mg/L) solution and it was irradiated by BLB lamps. A UV-Vis Spectrophotometer was used to estimate the concentration of the unreacted MB by measuring the attenuation at its absorption maximum of 660nm at 2h intervals up to 10h and the final measurement was taken at 24h.

2.3 Test Organism

The antibacterial properties of anodized surfaces were evaluated using Gram negative *Pseudomonas* sp., as the test organism. The reason for the selection of the above genus is that it has been identified as the major colonizer of fresh water biofilms [10,11]. Characterization and identification of the bacteria up to genus level was carried out based on morphological, physiological and biochemical tests as per Bergey's Manual of Systematic Bacteriology[12].

2.4 Exposure of Specimens

Exposure studies were conducted in a cylindrical glass vessel containing the respective exposure medium. A glass rod positioned centrally in the glass vessel supported the glass pegs, which bore the coupons. The specimens were illuminated by six numbers of black light blue (BLB) lamps (4 W, Philips) arranged in a hexagonal configuration surrounding the test vessel. The light produced by BLB lamps has wavelength range of 350-380nm and hence is referred to

as near-UV light to distinguish it from the UV light used normally for disinfection. The near-UV light used in the study is not having any bactericidal property and it is transmitted through ordinary glass. Therefore, no quartz vessel was used for the study. A dilute nutrient culture was prepared by inoculating 1% (0.13g/L) nutrient broth with 0.1ml of 24h culture of *Pseudomonas* sp. in 100% nutrient broth. This culture in dilute nutrient medium was recultured and used for exposure studies. This dilute nutrient culture was used to avoid pelagic growth of bacteria and to favor biofilm formation. The culture was mixed uniformly in an orbital shaker and incubated for 12h at 32^{o}C before the coupons were introduced. The density of *Pseudomonas* sp. in the exposure medium (500ml) was 6 x 10^{8} cfu/ml. The exposure studies were conducted for a duration of 100h.

2.5 Post Exposure Analysis

Three coupons of each experimental condition (triplicate experiments) were used for total viable count (TVC) estimation[13]. The coupons were removed from the medium and gently washed to remove loosely adhering cells and the bacterial cells on the coupons were dispersed into 15ml sterile phosphate buffer (0.0425g KH_2PO_4, 0.19g $MgCl_2$ per litre) by ultrasonication for 5min. The length of sonication for optimum recovery of cells was found to be 5min. The ultrasonically cleaned surfaces were stained and observed to ensure complete recovery of cells. Serial dilutions of the bacterial cell suspension were prepared and 0.1ml of each dilution was plated onto Nutrient agar. The plates were incubated for 24-48h at 32^{o}C and the number of colonies counted. Mean TVC values were calculated for each coupon and the results are expressed as colony forming units (cfu) per cm^{2}.

Two coupons of each experimental condition exposed in the nutrient culture were used for direct microscopic observation. The coupons were gently washed with sterile water and air-dried in a sterile chamber and the coupon surface was flooded using acridine orange (0.1% solution in distilled water). After 2min, the excess stain was drained off and the coupons were washed in sterile water, dried and observed. Acridine orange, a fluorescent dye, differentially stains single stranded RNA and double stranded DNA, fluorescing orange when intercalated with the former and green while complexing with the latter[14] when observed under a Nikon Eclipse E600 epifluorescence microscope (excitation filter BP 490; barrier filter O 515). Thus, the number of orange fluorescing cells depict the actively metabolizing cells on the anodized and acid pickled titanium surfaces and the green fluorescing cell represent the photocatalytically inactivated microbial cells.

2.6 Growth kinetics of the Normal Vs damaged cells

A 48h growth curve of the bacterial cells collected from the titanium surfaces was plotted using turbidity and plate count method as well in order to assess the extent of damage incurred to the bacterial cells. The bacterial cells from the exposure medium both under light illuminated and dark conditions and from acid pickled and anodized surfaces were ultrasonically removed and 0.1mL was inoculated into 1% nutrient broth in test tubes. The growth behavior of these cells were studied by measuring the cell density at every 2h intervals upto 12h after which final readings were recorded at 24h and 48h. The cell density was determined by measuring the TVC using pour plate method as well as by measuring the turbidity of the cultures by measuring the attenuation at 600nm using a spectrophotometer.

3. RESULTS

3.1 Surface Analysis

The type of oxide formed on the titanium surfaces on anodization was analyzed using GIXRD. The results showed that the coupons anodized for 10min and 1h did not show any peak of anatase. Coupons anodized for 24h and 48h at 30V, 50V and 100V showed peaks corresponding to 25.3 indicating the presence of anatase type of TiO_2. However, in the case of the anodized samples which were subjected to heat treatment it was observed that even the coupons anodized for 10min and 1h showed peaks of anatase.(Fig 1)

Coupons anodized for 24h and 48h showed excellent peaks corresponding to that of anatase phase of TiO_2. Apart from the expected anatase phase coupons anodized at 24h and 48h also showed peaks signifying the presence of rutile phase corresponding to 2θ angle of 27.446 . (Fig 2). It was also observed that the peak intensity increased with increasing holding time as a result of heat treatment.

The oxide morphology and structure was characterized using Atomic Force Microscopy (AFM). The AFM images showed that the oxide growth became uniform with increasing anodizing time. The particle sizes as determined by the AFM study increased with increasing anodizing voltage, the coupons anodized at 30V had particle sizes ranging from 200–300nm, coupons anodized at 50V, 500-600nm and coupons anodized at 100V showed a wide distribution of particle sizes ranging from 150-1000nm. (Fig 3a, b, c)

Although the AFM studies on the heat treated anodized surfaces did not show evident morphological variation, it was observed that the particle size and surface roughness of the oxide film remained similar to the untreated samples in case of all the test surfaces.

3.2 Evaluation of Photocatalytic Activity

The photocatalytic activity of the anodized and acid pickled titanium surfaces was evaluated by methylene blue degradation method. The results showed that the photocatalytic activity as shown by the degradation of the methylene blue dye degradation was maximum on the anodized surfaces as compared to the acid pickled surfaces. The results from the comparative study conducted using the coupons anodized at 30V, 50V and 100V for 10min, 1h, 24h and 48h confirmed that maximum photocatalytic activity was indeed exhibited by the titanium surfaces anodized at 30V for 48h followed by 30V 24h, 50V 48h and 100V 48h. (Fig 4)

3.3 Evaluation of the photocatalytic inhibition of bacterial attachment

3.3.1 Photocatalytic activity Vs anodizing voltage

Studies on bacterial attachment conducted using *Pseudomonas* sp. for durations of 100 hours showed significant reduction in bacterial attachment on the anodized surfaces. The acid pickled surfaces were observed to have increased number of viable bacterial cells expressed in terms of colony forming units (cfu/cm^2). A comparative study involving the photocatalytic inactivation of bacterial cells on titanium surfaces anodized at three different voltages namely 30V, 50V and 100V showed that the surfaces anodized at 30V showed maximum photocatalytic activity as shown by the significant reduction in microbial attachment compared to the coupons anodized at 50 and 100V. (Fig 5)

Direct acridine orange counts (DAOC) confirmed a similar trend in the photocatalytic inhibition of microbial attachment on various anodized titanium surfaces. Maximum bactericidal activity was observed on the 30V 48h anodized coupons. In the case of 50V and 100V anodized coupons, also it was seen that photocatalytic activity increased with increasing anodizing time (Fig 6).

3.3.2 Photocatalytic activity Vs anodizing time
Titanium surfaces anodized for 10min, 1h, 24h and 100h were tested for bacterial inhibition and it was seen that the photocatalytic activity increased with increasing anodizing time irrespective of the voltage involved. Among the various titanium surfaces maximum photocatalytic activity was recorded on surfaces anodized at 30V-48h followed by those anodized at 50V and 100V for 48h. (Fig 5,6).

3.3.3 Photocatalytic activity Vs heat treatment
Heat treatment increased the stability of the oxide film, making it extremely adherent to the substrate. The traditional pickling process employed for removing oxide formed on anodization failed to remove the oxide formed after the anodized surfaces were subjected to heat treatment. With respect to bacterial attachment, heat treated surfaces were less prone to bacterial attachment. It was also observed that heat treatment increased the photocatalytic activity of the otherwise slightly less active 100V and 50V surfaces. The heat treated 30V surfaces showed extremely significant photocatalytic activity. (Fig 7)

3.4 Growth kinetics of normal Vs photocatalytically damaged cells
The results of the 48h growth curve showed that the bacterial cells from the exposure medium as well as cells removed from acid pickled surfaces entered the log phase even by 2h and were actively increasing with time. However, in the case of the cells removed from the anodized surfaces, the bacterial cells remained in the lag phase even upto 8h after which they gradually entered the log phase and eventually the stationary phase (Fig 8).

4. DISCUSSION

Photocatalytic activity (PCA) is the ability of a material to catalyze oxidation/reduction reactions on illumination by light of suitable wavelength. Most of the semiconducting oxides exhibit this property. When such materials are illuminated with light of appropriate wavelength, electron-hole pairs are produced in the oxide by the transfer of a valence band electron to conduction band. Photocatalytic activity strongly depends on the surface redox potential, the band-gap and the lifetime of photo-generated electron hole-pairs. Anatase, which has a larger band gap, tends to increase the surface redox potentials and prolong the carrier lifetime in comparison with rutile[15]. Hence, anatase is a more efficient photocatalyst compared to other forms of titanium oxide such as rutile and brookite.

Earlier studies conducted in our laboratory comparing the effect of anodizing voltage on photocatalytic activity using coupons anodized at 30V, 50V and 100V for 10min showed that the photocatalytic activity did not increase with increasing voltage[16]. Photocatalytic activity was thus identified to be a complex function of physical characteristics of the oxide and a number of factors seemed to be involved. The aim of this study was to study the influence of the anodizing voltage, anodizing time and heat treatment on photocatalytic activity, in order to identify which of these factors could be possibly manipulated to achieve maximum reduction in microbial attachment (enhance photocatalytic activity).

The results from the experiments comparing coupons anodized at 30V, 50V and 100V for 10min, 1h, 24h and 48h have shown that photocatalytic activity did not increase with increasing voltage but did increase with increase in anodizing time. The coupons anodized at 30V for 48h seemed to exhibit maximum photocatalytic activity compared to the coupons anodized at 50V and 100V for 48h. The GIXRD results showed that the intensity of the anatase peaks were more in case of the coupons anodized at 30V for 48h, the intensity of anatase in case of 50V and 100V was also seen to increase with increasing anodizing time. Birch and Burleigh have reported an increase in oxide thickness of about 2nm for every volt[17]. Further evidence can be

quoted from our earlier work where coupons anodized at 30V, 50V and 100V for 10min were reported to have a thickness of 60nm, 100nm and 200nm respectively using ellipsometry[16]. Yu et al discussing about the influence of oxide thickness on photocatalytic activity, have reported that specific photocatalytic activity decreases with increasing thickness and that photocatalytic activity depended on many factors, such as, the distance to which the reactant should reach to capture the electrons of the holes generated in TiO_2 thin films, the amount of hydroxyl ions per unit weight TiO_2, film thickness, average grain size and so on[18]. This possibly explains why the photocatalytic activity did not increase with increasing oxide thickness.

Regarding anodizing time, not much literature is available, from the data evolved from this study it can be said that with increasing anodizing time the crystallinity of the oxide particles were on the increase as shown by the GIXRD results where the anatase peaks became more sharper with respect to time. According to Chen et al[19] the sharper the peaks higher is the crystallinity of the oxide particles. High crystallinity minimizes the photoexcited electron-hole recombination rate thereby enhancing photocatalytic activity[20] this seems to be the reason why the surfaces anodized for longer time periods showed high photocatalytic activity.

Heat treatment favored the formation of both anatase and rutile phases whose band gaps are 3.23 and 3.02 eV respectively. Generally it is accepted that anatase titania is more efficient as photocatalyst than rutile which has a lower band gap. However, our results showed that the occurrence of the rutile phase along with the anatase phase enhanced the photocatalytic activity of the test surfaces. Some researchers [21] have demonstrated that catalysts with mixed phases possessed significantly higher catalytic activity than the pure anatase phase. Degussa p-25 a standard industrial photocatalytst, which is composed of 70% anatase and 30% rutile, is a good example. The main reason is ascribed to better charge carrier separation in the mixed phase. Heat treatment was seen to enhance the formation of anatase phase on surfaces anodized 10min and 1h also whereas in the case of the untreated samples, GIXRD failed to give any peaks of anatase indicating that the anatase formed was amorphous or considerably less to be efficiently detected. Future studies are planned to study the effect of various temperature ranges on photocatalytic activity.

Mechanism for the bactericidal action of TiO_2 has been proposed by a number of authors and reported by Blake et al[22]. The valance band hole is capable of oxidizing water to form hydroxyl radicals in water[23]. Thus, hydroxyl radicals are believed to play a vital role in cell oxidation in aqueous systems such as ours where water is the major interface since the coupons are exposed in water. Yu et al[24] have also reported that hydroxyl ions exist only on the surface of thin films supporting the observation that photocatalytic activity does not increase with increasing thickness of the oxide.

Studies on the growth kinetics of the normal bacterial cells and the photocatalytically inactivated bacterial cells showed that the latter underwent a prolonged and abnormal lag phase. The lag phase is the period during which the cell is getting ready to divide, synthesizing all its cellular requisites like protein, enzymes, protoplasm, cell phosphorous etc. The prolonged lag phase indicates that the cell had to repair the damages suffered due to the free radical activity before they could normally divide as the other cells. A similar trend, where a longer time was needed by *E.coli* cells to regain their growth capability before entering the log phase has been reported [25]. A two-order difference between the total viable counts determined by plate count method and direct acridine orange counts was observed throughout this study. This indicates that some of the cells, which have been inactivated and not fluorescing orange and thus were not included in the DAOC, were able to grow on agar plates, indicating cell membrane attack. Cell membrane is the limiting membrane of the bacterial cell and therefore the primary site of attack of any reactive species is the cell membrane[26]. The bacterial cell membrane provides an attachment site for cellular respiration[27]. Hence, any damage to the cell membrane results in loss

of respiratory activity in the cell, loss of respiratory activity would mean no production of energy yielding ATP molecules. This would lead to a total decrease in the metabolic rate of the bacterial cell, thus explaining the 6h long abnormal lag phase or low metabolic state the damaged cells experienced, as given by the results of this study. It has been also observed that there is a marked delay in the development of such colonies on the agar plate, depicting a primary set-back for the cells to repair damages. The combination of cell membrane damage, and further oxidative attack of internal cellular components, ultimately results in cell death[22]. Oxidative attack of the cell membrane leads to lipid peroxidation[28]. The results obtained comparing the Gram negative bacteria which possess a lipoproteinaceous outer membrane and the Gram positive bacteria lacking the outer membrane have also supported the argument that lipid peroxidation of the cell membranes during free radical attack is one of the major reason for cell death [29]. Although the above reasoning explains the photocatalytic inhibitory effect of TiO_2 on the growth of bacteria, detailed investigations quantifying the growth kinetic parameters of bacterial cells damaged by photocatalysis will help elucidate the damage mechanism.

5. CONCLUSIONS

1. Surface studies involving GIXRD identified the type of oxide to be that of anatase GIXRD results also showed a gradual increase in the intensity of anatase with increasing anodizing time but not with voltage. On heat treatment crystalline anatase as well as rutile phases were formed. AFM results showed that the surface oxide was uniformly grown all over the surface in case of 30V anodized surfaces whereas in 100V anodized surfaces a number of voids and varying range of particle sizes was observed. Heat treatment did not affect the particle size nor the surface roughness of the oxide films.

2. The photocatalytic activity of the surfaces anodized at 30V, 50V and 100V for 10min,1h, 24h and 48h was evaluated using dye degradation methods and by antibacterial studies using *Pseudomonas* sp. Of all the surfaces studied it was seen that the titanium surfaces anodized at 30V for 48h exhibited maximum photocatalytic activity. The results showed that photocatalytic activity did not increasing with increasing voltage (thickness) but did increase with anodizing time.

3. Heat treatment was observed to increase the stability and enhancing the photocatalytic activity of the oxide film formed on the titanium surfaces by anodizing.

4. Growth kinetics of photocatalytically deactivated cells, compared to normal cells showed a delay in reaching the exponential or dividing phase, suggesting the extent of damage incurred to the cells. The differences in the plated counts (TVC) and the direct acridine orange counts suggest membrane damage to be the cause of cell inactivation.

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FIGURES

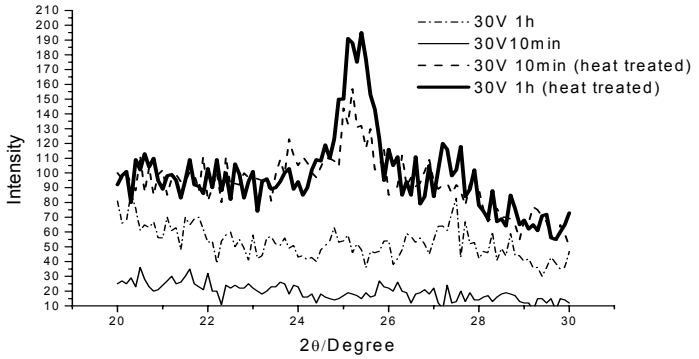

Fig 1; GIXRD results comparing coupons anodized at 30V for 10min and 1h in the presence and absence of heat treatment

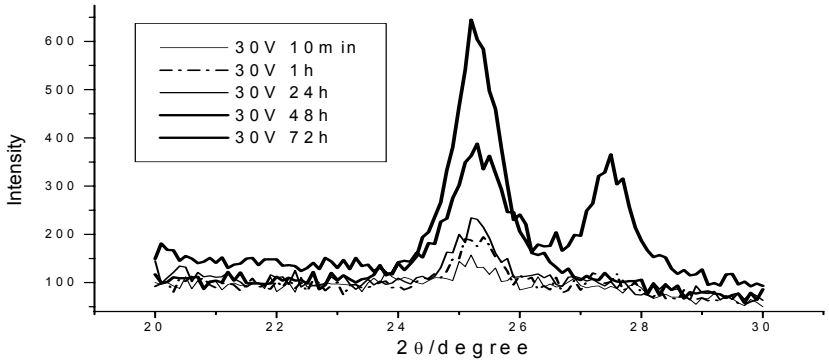

Fig 2: GIXRD results of titanium surfaces anodized at 30V at different anodizing times followed by heat treatment

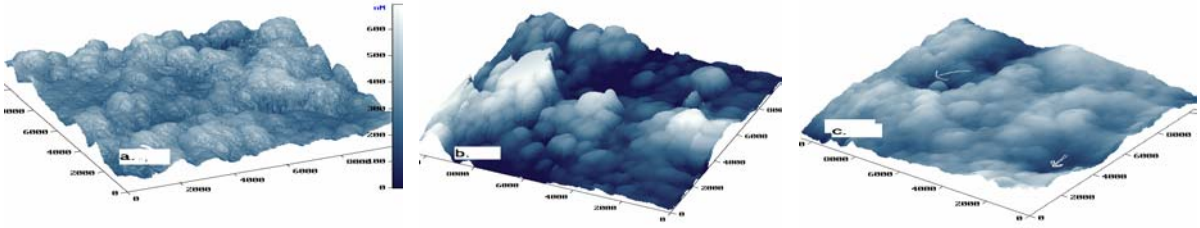

Fig 3: AFM images showing oxide grown on a). 30V- 48h b). 50V- 48h c). 100V- 48h anodized titanium surfaces prior to heat treatment

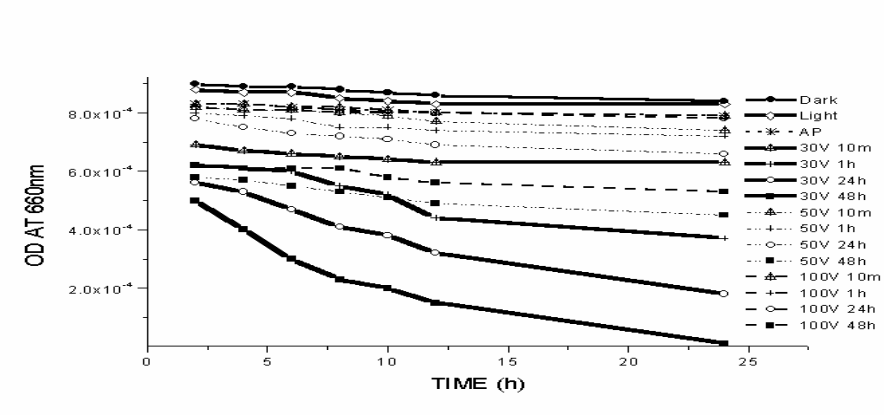

Fig 4: Photocatalytic dye degradation of Methylene blue dye by the various test surfaces

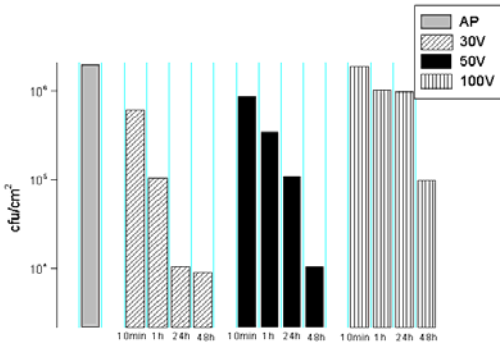

Fig 5: TVC of *Pseudomonas* on the surfaces anodized at 30V, 50V and 100V for 1h, 24h and 48h and acid pickled surfaces

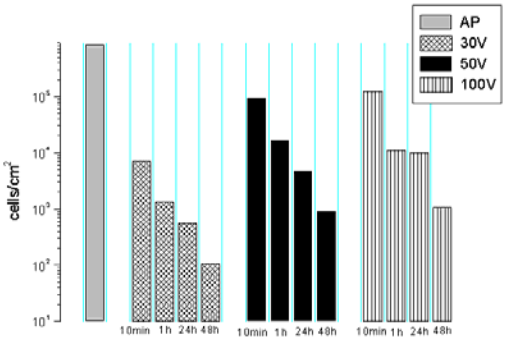

Fig 6: DAOC of *Pseudomonas* on the surface anodized at 30V, 50V and 100V for 10min 1h, 24h and 48h and acid pickled surfaces

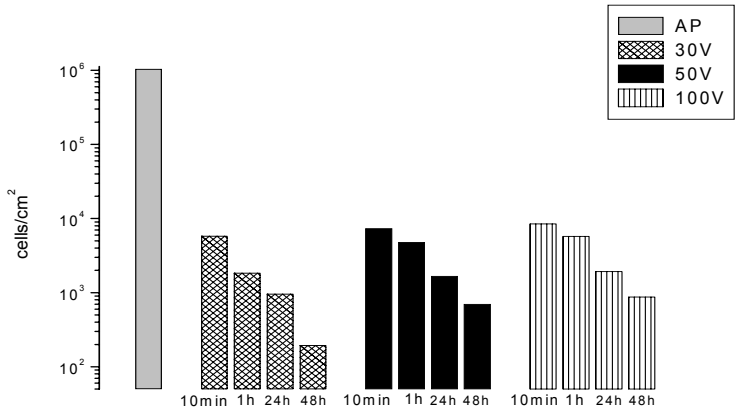

Fig 7: DAOC of *Pseudomonas* on the surfaces anodized at 30V, 50V and 100V for 10min, 1h, 24h and 48h showing the effect of heat treatment on photocatalytic activity

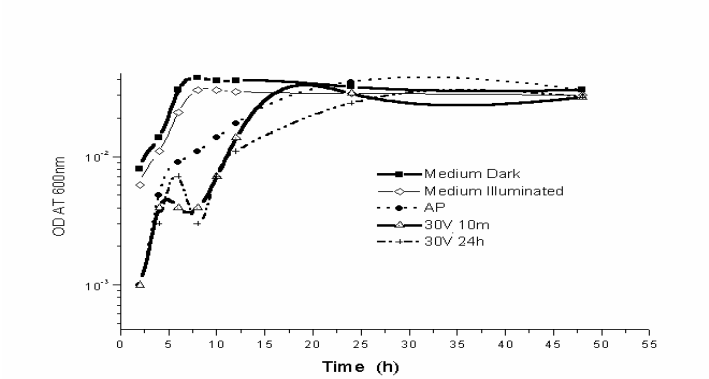

Fig 8: Growth curve comparing the growth of normal and photocatalytically affected bacterial cells