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Electrolytic Stimulation of Bacteria Metabolism

Bo Song*, Yun Tang, Yi-Ling Hou and Xiang Ding

College of Life Science, China West Normal University, Nanchong, Sichuan - 637 002, China.

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The effects of a weak electric field on the growth and metabolic behavior of *E. dissolvens* were investigated using glucose as the sole carbon source. A Alternating current (AC) was applied using salt bridge electrodes and platinum electrodes. When 10 mA current were applied water elctrolysis was not induced in the process of AC stimulation.The best stimulating effects in terms of cell growth and the dehydrogenase activity(DHA) were obtained when a AC of 100mA was applied for 12 hour via the platinum electrodes. In this case, the electrolysis of water was the major electrode reaction, as determined by cyclic voltammetry. The presence of the hydrogen generated a strong reductive environment and led to a reduction environment. The specific activity of dehydrogenase and glucose consumption increased 1.4 and 2.17 fold, respectively. The application of the AC via the platinum electrodes also led to accelerated cell growth during the later stationary phase. This is possibly due to the presence of anodic intermediates including H_2O_2, $OH^{\cdot}$ and $O_2^{\cdot}$. These results provide more details for understanding the effect of a AC on *E.* dissolvens, a strain with potential application in the electro-remediation of PAHs contaminated soil.

Key words: Electrostimulation, Alternating current, Dehydrogenase activity, Glucose, Redox potential.

Pulsating (pulsatile) electromagnetically induced currents (PEMIC, PEMF, PMF),simple alternating currents (ac) and even direct currents (dc) were applied to cells,tissues and organisms in order to stimulate membrane permeabilization and some pathways of the metabolism for the first time about the last century .Several controversial reports claiming important bioeffects of different electric fields on celluar systems have been published partly due to the different experimental conditions and also to biological in biosciences. Electctrolytic stimulation refers to a microbial process performed in the variability,nevertheless some important morphological and biochemical changes were emphasized pointing out a new fundamental tool in future research .

* To whom all correspondence should be addressed.
E-mail: songbo1@126.com

In presence of electrolysis by a weak direct current (DC) or Alternating current(AC). The exposure to a 100mAAC may lead to an enhanced fermentation of yeast[1] and the reduction of nitrate[2,3]. The current intensity for the electrolytic stimulation process is normally kept below 20 mA to avoid the bactericidal effect observed at high intensity[4], However, negative effects of weak DC have also been reported, such as the inactivation of *T. ferrooxidans and Acidiphilium SJH* in liquid culture[5] and the enhancement of antibiotic efficiency against a wide variety of biofilms[6-8]. These diverse results indicate the complexity of the electrolytic stimulation process and growing at the tention directed to the fundamental study of the mechanism of electrolytic stimulation. Nakanishi *et al.* have suggested that the improved yeast growth and ethanol production is due to the oxygen generated at the anode[1]. Beschkov *et al.* assumed that the hydrogen generated at the

cathode was responsible for the enhanced bacterial denitrification[3]. Despite the many efforts to increase the understanding of the mechanism of electrolytic stimulation, a more comprehensive investigation on diversified microbiological systems that focuses on cell metabolic behavior in an electric field is still needed.

Our aim is to apply AC for electrostimulation of micro-organisms in a laboratory-scale culture

MATERIALS AND METHODS

Bacterium and chemicals

A gram-negative bacillus strain capable of utilizing phenanthrene as the sole carbon source was isolated from the petroleum-contaminated soil sampled in Tianjing, China. The strain was identified to be *Enterobacter dissolvens*. Phenanthrene and TTC as 99.0% analytical standards were both purchased from Alfa Aesar (Massachusetts, USA). Other reagents used are all in analytic grade.

Medium and pre-cultivation

The strain was pre-cultivated in shaking flask to multiply cell mass. The culture medium was prepared by mixing glucose with basal mineral medium containing, which finally contained (in g/l deionized water): glucose (6.7 g/L), K_2HPO_4 (0.8), KH_2PO_4 (0.2), NH_4NO_3 (0.8), $MgSO_4$ (0.25), $FeSO_4 \cdot 7H_2O$ (0.09), and $CaCl_2$ (0.032). Pre-cultivation was started by adding 0.1 ml of acclimatized inoculum into 150 ml of culture medium in shake flask (300 ml), and proceeded at 30°C (by shaker, 150 rpm) for approximately 10 hours when cell growth just entered the early log phase (0.2~0.3 in OD_{600} against fresh medium). The culture was then taken out and subjected to electrolytic stimulation experiments.

Electrolytic stimulation

The electrolytic stimulation experiments were conducted in two identical jacketed CSTR-type vessels (working volume: 100 ml) as shown in Peng She[9]. Two electrodes made of either platinum (Pt) wires (0.3 mm diameter) or agar bridges (1 mm tip diameter, 1.7% of agar and 30% of KCl in basal medium) were installed on one vessel to implement AC current. The other vessel was then used for the control experiment without AC current. Before the electrolytic experiment, the two vessels were both autoclaved and then filled with 70 ml of pre-cultivated cell culture in each one. During the experiments, the broths were continuously cultivated on a multi-heads magnetic stirrer with the same mixing intensity. AC current was supplied by an electrophoresis apparatus (Liuyi Instrument, Beijing, China) and thermostatic water at 30°C continuously flowed through the jackets of both vessels. Culture samples were periodically taken out from each vessel, then immediately subjected to further analysis.

Analysis

Dehydrogenase activity

The dehydrogenase activity was measured by using 2,3,5-triphenyl tetrazolium chloride (TTC) as a terminal acceptor of protons and electrons from glucose being oxidized. After sampling 2 ml of the cell suspension was immediately mixed with 2 ml of glucose solution (0.1 M), 2 ml of Tris-HCl buffer (pH 8.4) and 2 ml of TTC solution (0.5%). After 3 hours incubation at 30°C, the mixture was extracted by 5 ml of toluene. The 2,3,5-triphenyl formazan (TPF) product in the organic phase was then spectrometrically measured at 490 nm against toluene. After divided by the corresponding cell density value (OD_{600}), the OD_{490}/OD_{600} ratio was directly used as an index to the specific dehydrogenase activity.

Cell density and glucose concentration

Cell density in culture solution was determined by the optical density at 600 nm, and also checked by colony counting on agar plate in case of necessity. Glucose concentration was analyzed by dinitrosalicylic colorimetric (DNS) method [10]. Electrolysis test in fresh medium confirmed that glucose is stable in the presence of electrode reactions (data omitted).

Scanning electron microscopy

The sampled cell culture (1 ml) during the experiments was immediately fixed with glutaraldehyde (3% aqueous solution) and stored at 4°C. Before subjected to scanning electron microscopy (JSM-6330F, JEOL, Japan), cell samples were pretreated by critical point drying to maintain the original morphology.

Electrode hydrolysis reaction intermediate product analysis

Electrolytic stimulation process OH- (hydroxyl free radicals) and H_2O_2 (hydrogen peroxide) The testing of the two hydrolysis

intermediate product is according to Wabner provide method. Its principle is to use to nitroso dimethyl aniline (RNO) as capture reagent in neutral solution and OH - selective reaction, and make their own with yellow faded.This change can be measured by the solution in the 440 nm in absorbance (OD_{440}) for real-time monitoring. H_2O_2 is use plus Fe^{2+} ion into OH^- Fenton reaction[11] also through the monitoring solution to determine OD_{440} change.

Redox potential and pH

The redox potential and pH of culture solution were off-line measured by a combined platinum-redox electrode (Pt4865-50-S7, Mettler Toledo, Switzerland) and a pH sensor (Z71201AP40, Applikon, the Netherlands), respectively.

RESULTS AND DISCUSSION

Effects of AC current in the absence of electrode reaction

During the electrolytic stimulation process, the influences of AC current on bacterial cells should be characterized in terms of two factors: pure current and electrode reaction. The contribution from pure current was first evaluated by monitoring cell growth and glucose utilization with 10-mA current implemented by agar-bridge electrodes. In this case the interference from electrode reactions was completely inhibited. As shown in Fig. 1, thereis no increases in cell growth and glucose uptake were observed, Therefore, the influence of pure current on cell growth is limited . This conclusion is also in accordance with the

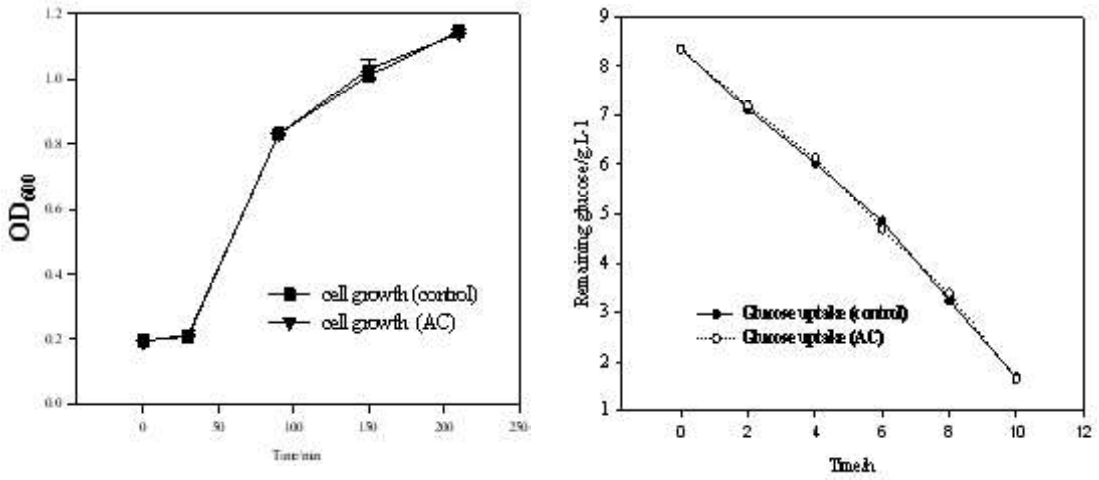

Fig. 1. Cell growth (a)and glucose uptake(b) under AC current implemented via salt bridge (Current intensity: 10 mA) and in the control run

conventional viewpoint, that pure AC current has no effect on the bacteria in inducing electrostimulation effects.

Cell growth and glucose uptake in electrolytic cultivation process

Mediated by a large group of intracellular dehydrogenases, the catabolism of organic compounds by heterotrophic bacteria is essentially a dehydrogenation process, in which protons and electrons were generated from the substrate and then transported to the finial acceptor via the intracellular electron transport chain. Therefore, the dehydrogenase activity (DHA) was often used to represent the microbial activity. In order to characterize the effects of water electrolysis on cell growth and glucose uptake, electrolytic stimulation experiments with AC current at various intensity were conducted. Changes in cell DHA, glucose concentration and cell growth were measured in both electrolyzed culture and the control culture as a function of time.

10mA current were applied ,Water electrolysis was not induced in the process of AC stimulate. each parameter of the bacteria is not obvious change (data is omitted) Fig. 2(a)and(b) represent the experimental results obtained at 100

mA current. As shown in figure 2(a), notable increases in cell DHA and glucose uptake were obtained in electrolyzed culture. After 12 hours exposure, the specific DHA represented by TPH(OD_{490})/Cell density (OD_{600}) had reached 2.17 times in electrolyzed culture over the control value, and 1.4 times in terms of the glucose uptake. Besides the relevancy between glucose utilization and cell DHA, present results clearly indicated glucose metabolism was enhanced in the presence of electrolytic reaction. And the Cells is in the stationary phase growth(a), glucose uptake(b), during the electrolytic cultivation via Pt electrodes and in the control run (Current intensity : 100mA)

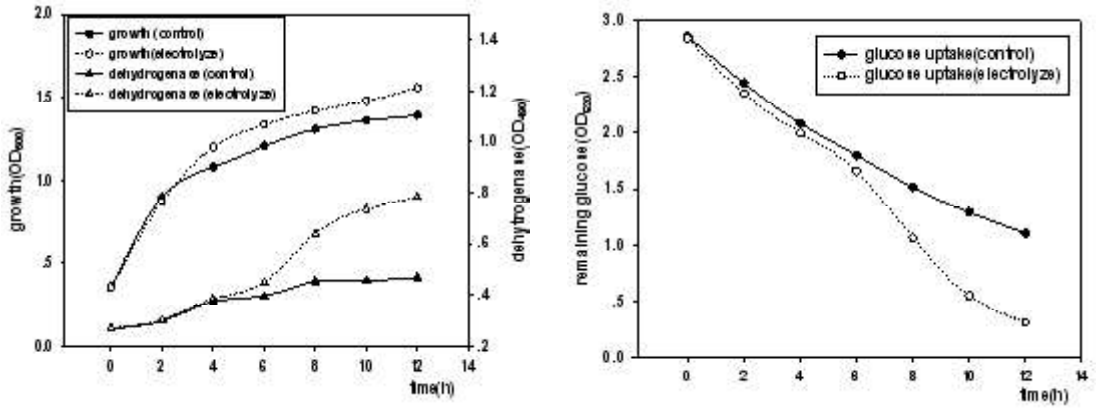

Fig. 2. Time course profiles of the cell dehydrogenase activity and cell

The influence from electrolysis reaction by AC current was also indicated by the changes in solution properties. During all the AC electrostimulation experiments presented above, there is visible changes in solution pH Changes in the physicochemical properties (pH and redox potential) of culture solutions were simultaneously recorded and shown in Fig. 3. The culture pH continuously ascend during the cultivation process, indicating the capability of producing organic alkali by the strain. Furthermore, no apparent distinction in pH was observed on both cultures within the first 12 hours. Although a 1 difference in pH appeared at the end of the experiment, this change was obviously not enough to fully account for the greatly accelerated ascend of cell DHA shown in figure 2(a). On the other hand, the implementation of AC current also led to an immediate decrease in the redox potential (Eh) of electrolyzed culture, which is always below -200 mV and much lower than the control value. This indicates the presence of water electrolysis maintained a strong reductive environment during the electrolytic cultivation process, which is probably the reason for enhanced cell DHA and glucose utilization.

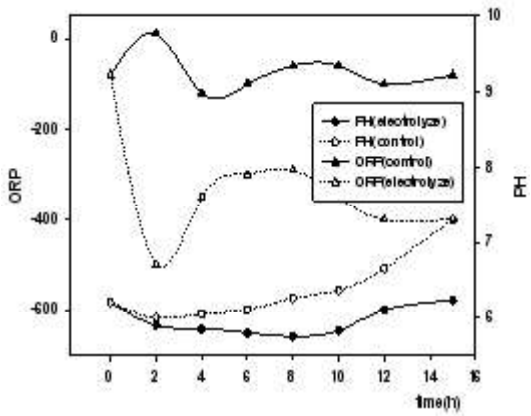

Fig. 3. Time course profiles of the cell polution properties during the electrolytic cultivation via Pt electrodes and in the control run (Current intensity : 100mA)

The dependence of microbial DHA on environment reducibility had been extensively observed in soils[10-12]. However, to our best knowledge there is no report pointing out water electrolysis reaction can be utilized to shift the redox environment, and improve the cell DHA. According to the voltammegrams shown in figure 4, and referring to the research on electrolyzed–reduced water by Sanetaka, *et al*[13], the decline in

solution Eh pertained to the hydrogen generated by water electrolysis on cathode. As a proton donor, hydrogen may stimulate the dehydrogenase activity, and then improve the utilization of glucose.

As two oxidative radicals, $OH^{\bullet}$ and $O_2^{\bullet}$ had been reported to be potentially harmful to bacterial cells[14]. H_2O_2 is also a well-known bactericide, and its destructive effects on cell

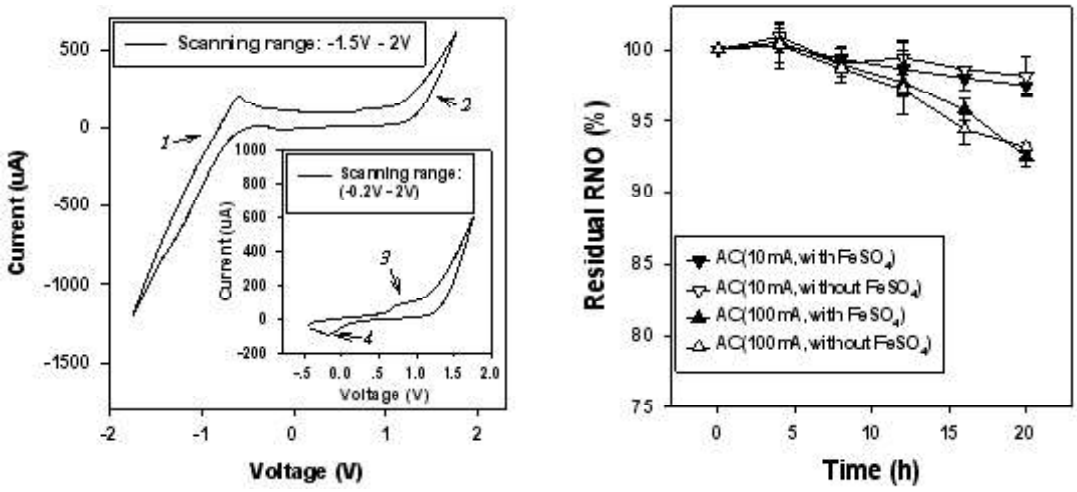

Fig. 4. Cyclic voltammegrams of Pt electrode in cell culture

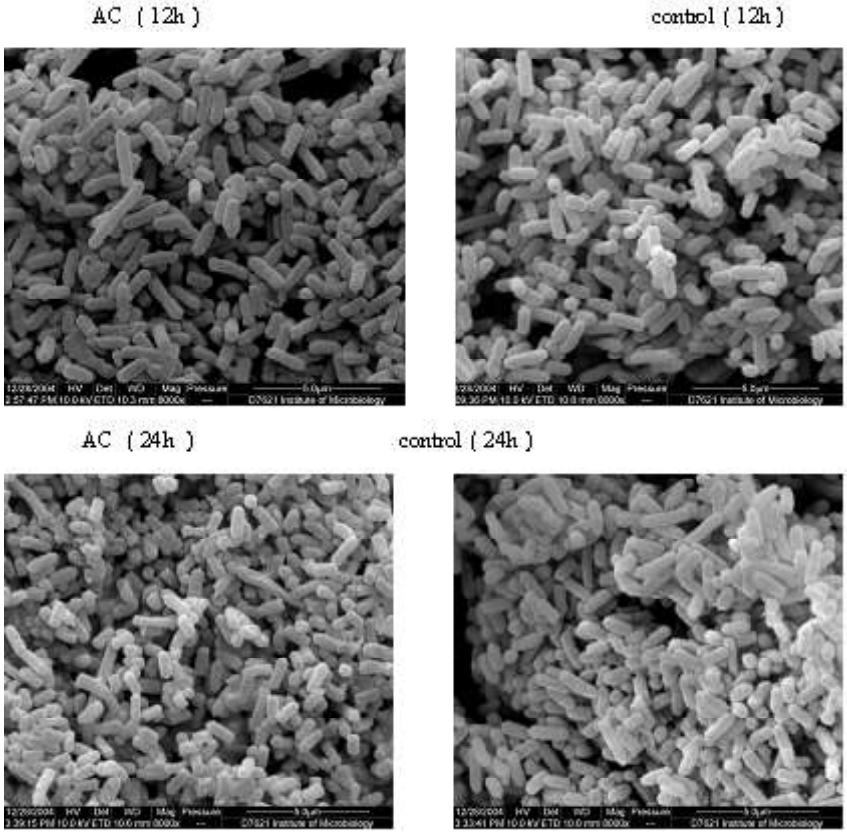

Fig. 5. Morphology of the electrolyzed cells and the cells in control run after different culture time (magnification: X8000)

membrane[15] is exactly corresponding to the morphological changes on electrolyzed cells. In this regard, the current intensity used should be optimized to minimize the bactericidal effect of water electrolysis during the electrolytic cultivation. Based on the experimental observation, AC current at 100mA was much more applicable in comparison with currents at 10 mA in present experimental system.The enhanced activity of extracellular $O_2^{\bullet}$ demonstrates clearly the effect of electrostimulation on cell metabolism.

Cell growth in electrolytic cultivation process

In the 100 mA experiment cell growth was characterized by both colony counting method and measuring the optical density at 600 nm. Fig. 2 (a) shows a correspondence match between the results obtained by both means. As a response to the improved glucose uptake, apparent increase in cell density was obtained in electrolyzed culture in comparison with the control culture during the first 12 hours. To further examine the electrolyzed cells, culture samples from the 100 mA experiment were imaged by scanning electron microscopy. Two time points at the early stationary phase (12 h) and the late lag phase (24 h) of cell growth were included in sampling procedure. As shown in figure 5, cell integrity and surface morphology were well maintained in both cultures after 12-hour cultivation. meanwhile, The cellular structure keeping intact was observed in the electrolyzed culture after 24 hours exposure, indicating there have no obvious stimulation in AC applied field.

CONCLUSION

The electrolytic stimulation process of strain *E. dissolvens* was investigated in present research. Water electrolysis was identified to be the dominant electrode reaction and apparently enhanced the cell growth and glucose uptake. The increase in cell DHA pertained to the generation of hydrogen on cathode. In the stationary phase of cell growth, electrode reaction also led to ascend cell viability, preliminarily attributed to the anodic intermediates of water electrolysis. For practical application of the electrolytic cultivation, current intensity need to be optimized and a discontinuous electrolysis might be suitable for a long-term cultivation process.

The feasibility of stimulating the microbial activity by adjusting culture Eh had been reported in recent, in which salts including Ti(III) citrate was successfully applied as a reducing agent [16]. In contrast, the merits of electrolytic stimulation not only rely on the reducibility of water electrolysis reaction, but also consist of the advantages in operating cost and environmental safety. Further study will be concentrated in the influence of electrolytic stimulation on cell metabolism, and the feasibility of applying this process to the bioengineering.Such results are promising practical use .However.their unambiguous explanation is still a challenge for the future

REFERENCES

1. K. Nakanishi, H. Tokuda, T. Soga, T. Yoshinaga, M. Takeda, Effect of electric current on growth and alcohol production by yeast cells, *J. Ferment. Bioeng.* 1998; **85**: 250-253.
2. A.M. Hayes, J.R.V. Flora, J. Khan, Electrolytic stimulation of denitrification in sand columns, *Water Res.* 1998; **32**: 2830-2834.
3. V. Beschkov, S. Velizarov, S.N. Agathos, V. Lukova, Bacterial denitrification of waste water stimulated by constant electric field, *Biochem. Eng. J.* 2004; **17**: 141-145.
4. M. Diao, X. Y. Li, J. D. Gu, H. C. Shi, Z. M. Xie, Electron microscopic investigation of the bactericidal action of electrochemical disinfection in comparison with chlorination, ozonation and Fenton reaction, *Process Biochem*. 2004; **39**: 1421-1426.
5. S. A. Jackman, G. Maini, A. K. Sharman, C. J. Knowles, The effects of direct electric current on the viability and metabolism of acidophilic bacteria, *Enzyme Microb. Technol.* 1999; **24**: 316-324.
6. S.A. Blenkinsopp, A.E. Khoury, J.W. Costerton, Electrical enhancement of biocide efficacy against *Pseudomonas aeruginosa* biofilms, *Appl. Environ. Microbiol.* 1992; **58**: 3770-3773.
7. N. Wellman, S.M. Fortun, B.R. McLeod, Bacterial biofilms and the bioelectric effect, Antimicrob. *Agents Chemother.* 1996; **40**: 2012-2014.
8. P.S. Stewart, W. Wattanakaroon, L. Goodrum, S.M. Fortun, B.R. McLeod, Electrolytic generation of oxygen partially explains electrical enhancement of tobramycin efficacy against

Pseudomonas aeruginosa biofilm, *Antimicrob. Agents Chemother.* 1999; **43**: 292-296.

9. Peng She,Bo Song,Xin-Hui Xing etl.Electrolytic stimulation of bacteria *Enterobacter dissolvens* by a direct current.[J] *Biochemical Engineering Journal.*2006; **28**: 23-29
10. M. Brzezinska, Z. Stepniewska, W. Stepniewski, Soil oxygen status and dehydrogenase activity, *Soil Biology & Biochemistry*, 1998; **30**(13): 1783 – 1790.
11. LEI Lecheng ,SHEN Xueyou,HE Feng Mechanism of Photo-Fenton Degradation of ethanol and PVA.*Chinese journal of chemical engineering* .2003; **11**(5) :577-582
12. J. Glinski, Z. Stepniewska, M. Brzezinska, Characterization of the Dehydrogenase and Catalase Activity of the Soils of two Natural Sites with respect to the Soil Oxygenation Status, *Polish Journal of Soil Science*, 1986; **19**: 47-52.
13. S. Sanetaka, K. Shigeru, N. Mariko, M. Takumi, *et al*, Electrolyzed–reduced Water Scavenges Active Oxygen Species and Protects DNA from Oxidative Damage, *Biochemical and Biophysical Research Communications,* 1997; **234**(1): 269-274.
14. Diao M., Li X.Y., Gu J.D., Shi H.C., Xie Z.M., Electron microscopic investigation of the bactericidal action of electrochemical disinfection in comparison with chlorination, ozonation and Fenton reaction, *Process Biochemistry*, 2004; **39**(11) : 1421 – 1426.
15. T.Z. Liu, T.F. Lin, D.T.Y. Chiu, K.J. Tsai, A. Stern, Palladium or Platinum Exacerbates Hydroxyl Radical Mediated DNA Damage, *Free Radical Biology and Medicine*, 1997; **23**(1): 155-161.
16. G. Jin, A.J. Englande, Redox Potential as a Controlling Factor in Enhancing Carbon Tetrachloride Biodegradation, *Water Science and Technology*, 1996; **34**(10): 59.