Morfological alteration in *Bacillus subtilis* by electrolisys

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ABSTRACT

The water treatment is indispensable for public health. Traditionally the water treatment involves addition of chemical substances. Those substances can generate toxic compounds affecting water quality. The process using chlorine for desinfection promotes the formation of trialomethanes, which have carcinogenic properties. The electrolytic treatment is an alternative for the drinking water and wastewater disinfection. Besides the public health advantages, the electrolytic treatments do not need addition of any substances to the process. The goal of this work was to observe the electrolytic treatment effects in the *Bacillus subtilis*'s morphology. *B. subtilis*'s suspensions in phosphate buffer 0.2M in pH 7.2, with chlorine free, were electrolyzed using a carbon vitrified as cathode and platinum foil as anode. The platinum foil was covered by a dialysis membrane. It was applied 0.60A DC during 30 min. The electrolyzed and not electrolyzed suspensions were colored by Gram method and observed in optical microscopy. Also it was observed by scanning electronic microscopy. The electrolysis promoted alterations in the *B. subtilis*'s cell wall and the cell cytoplasm was released.

KEY WORDS: disinfection, *Bacillus subtilis*, carbon electrode, electrolysis, morphologic alteration.

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INTRODUCTION

Treatment of drinking water is essential to public health. According to the World Health Organization 80% of all diseases in third world countries derive from contaminated water (TOMINAGA; MIDIO, 1999). In treating water, disinfection guarantees the killing of pathogenic organ-

isms as well as other organisms that may have hazardous effects (PIROU et al., 1998; TOMINAGA; MIDIO, 1999; Lutey, 2000).

Traditional treatment of water includes the addition of chemical substances that may produce toxic compounds that affect the health of those that use the water. The most used method is chloration, which is the addition of free chlorine (Cl_2) or substances that release this substance in the water in order to promote disinfection (TOMINAGA; MIDIO, 1999). Although cheap, this method promotes the formation of compounds such as trialomethanes, which are identified as carcinogenic, besides other toxic effects (MATSUNAGA et al., 1992; TOMINAGA; MIDIO, 1999).

Electrolytic treatment can be an alternative in the disinfection of residual and supplying water. Besides the advantages for public health, the electrolytic treatment is a clean process since it does not require addition of substances (LUBICKI; JAYARAM, 1996) and can be used in industries of high technology that needs extreme pure water for its procedures.

Rosenberg et al. (1965) studying the effects of electricity in the growth of *Escherichia coli* observed that cellular division was inhibited in these organisms, which was attributed by the authors to the formation of platinum compounds during electrolysis.

Death of bacteria during electrolysis has been attributed to several factors: the generation of chlorine compounds such as gaseous chlorine and hypochlorite from chloride ion (PARELEUX; SICARD, 1970; STONER et al., 1982; PATERMARAKIS; FOUNTOUKIDIS, 1990); the evolution of O_2 and O_3 in the cathode (PATERMARAKIS; FOUNTOUKIDIS, 1990); formation of free radicals such as HO_2^{3-} and OH^- (PATERMARAKIS; FOUNTOUKIDIS, 1990; TOLENTINO-BISNETO; BIDOIA, 2000 a, b); reactions due to the direct charge transfer between the electrode and the bacteria (PATERMARAKIS; FOUNTOUKIDIS, 1990; NAKASONO et al., 1992, 1993; BRATFICH et al., 1999; TOLENTINO-BISNETO; BIDOIA, 2000 b); oxidation of cell substances such as the Coenzyme A (MATSUNAGA et al., 1992; NAKASONO et al., 1992; OKOCHI et al., 1999); the destruction of the citoplasmatic membrane or the pure increase in permeability and decrease in membrane selectivity (LUBICKI; JAYARAM, 1996; FRIENDRICH et al., 1998; LEE; TAI, 1999); rapid alteration in pH (TOLENTINO-BISNETO; BIDOIA, 2000 a); the reductive capacity of ferrous ion in contact with the microorganism (ANGELIS et al., 1998; BRATFICH et al., 1999); the cell lyses (LUBICKI; JAYARAM, 1996; LEE; TAI, 1999).

The death speed is independent from the initial concentration of microorganisms and it is directly proportional to the density of current or to the potential applied in the process (PATERMARAKIS; FOUNTOUKIDIS, 1990; BRATFICH et al., 1999; LEE; TAI, 1999).

The aim of this study was to observe the effect of the electrolytic treatment with carbon cathode on the *Bacillus subtilis.*

MATERIAL AND METHODS

Electrolytic Cell

It was used an electrolytic cell of 80mL with circulation of water linked to a termostatized bath. The temperature of the system was below 25˚C. As cathode it was used a sponginous glassy carbon electrode made by Tokai Carbon Co.® from Japan, measuring 3.80 x 1.70 x 1.30 cm. The effective area of reaction in the cathode was 148.6 cm^2. As anode it was used a polycrystalline platinum electrode from Aldrich (99.98% m/m) covered with a dialysis membrane. In addition, the membrane was filled with 2mL of 0.2M phosphate buffer, pH 7.2, and chlorate free. The covering of the electrode aims to prevent its effect in the disinfections.

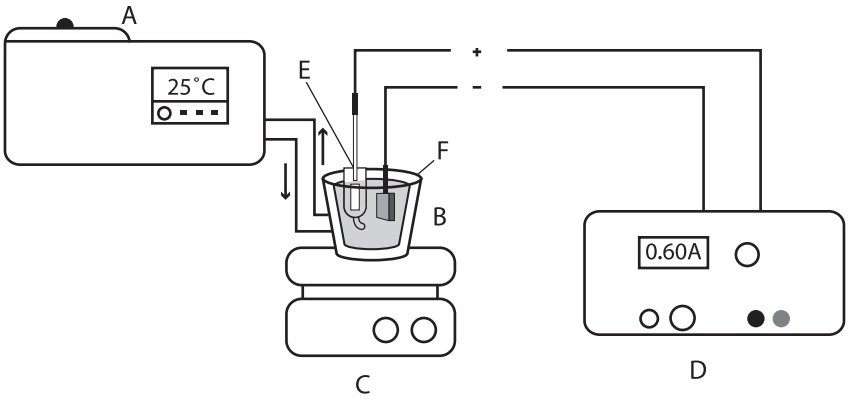


FIGURE 1: Scheme of the system of treatment. A: termostatized bath; B: electrolytic cell; C: magnetic stirring; D: DC power source; E: platinum anode covered by the dialysis membrane and F: carbon cathode.

Preparing the suspension

CCT 2576 cultures of *B. subtilis* were obtained from two consecutive steps. The first in a culture tube in solid nutrient Agar medium incubated at 28˚C for 24h. The second, in 100mL of liquid nutrient broth culture medium incubated at room temperature for 24h under stirring. Aliquots of 2mL from the liquid culture were added to 50mL of sterile 0.2M buffer phosphate, pH 7.2. To the electrolytic cell it was added 52mL of this suspension, which was constantly steered through the process by a magnetic stirring machine.

These suspensions were electrolyzed by continuous current of 0.60A for 30 minutes in a batchwise system. The pH and the temperature of the electrolyzed suspensions were recorded.

Light microscopy

Smear slides were made from the suspension of *B. subtilis* submitted or not to electrolytic treatment. The slides were stained by Gram (LAPERNT; GOURGAUD, 1975) and observed in light microscopy.

Scanning Electronic Miscroscopy

Treated and not treated suspensions of *B. subtilis* were fixed by glutharaldehyd, prepared (ALDRICH; TODD, 1986) and observed in the electronic scanning microscope.

RESULTS AND DISCUSSION

Light microscopy

B. subtilis cells not exposed to electrolytic treatment appeared as Gram positive rods easily observed in light microscopy.

After 30 minutes of treatment, cells appeared red stained by Gram (Gram negative) besides some modification in the form of the rods and diminution in size when compared to non-electrolyzed bacteria.

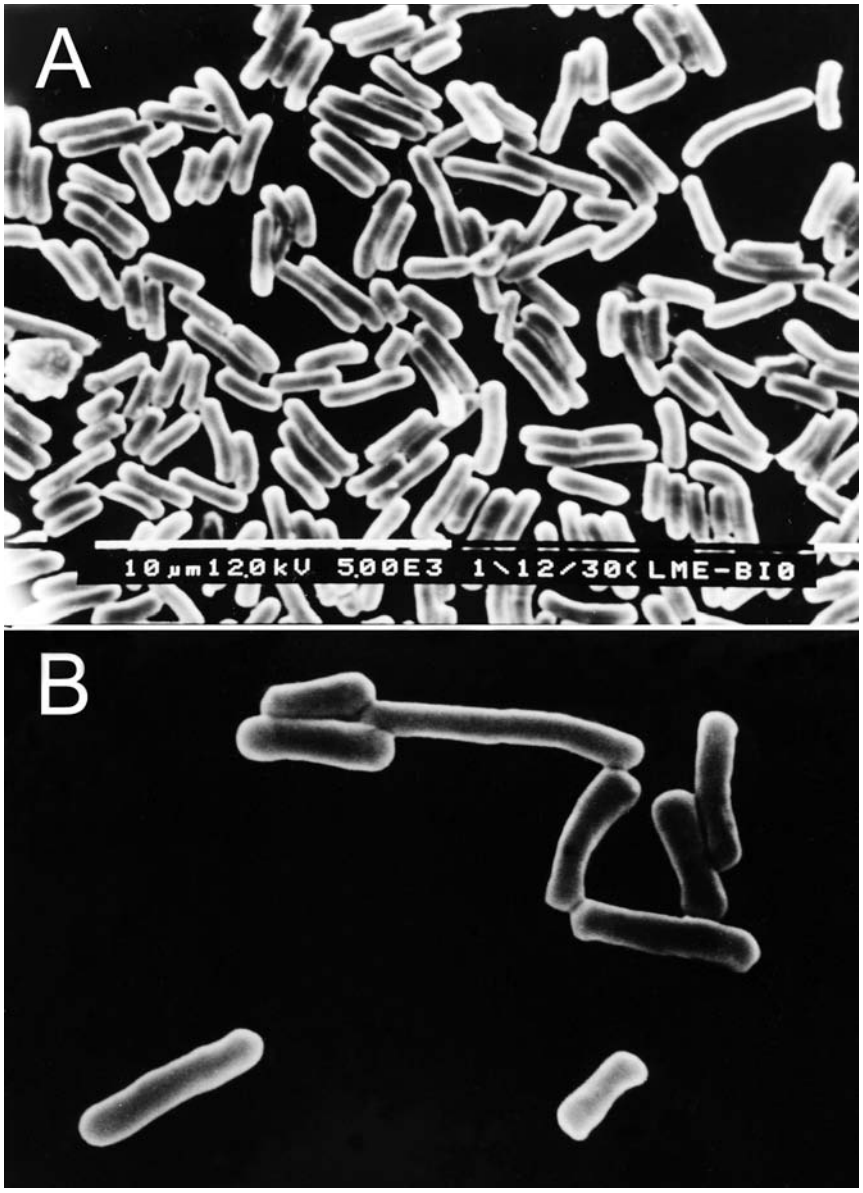
Results from light microscopy lead to the conclusion that electrolysis provoke alteration in the cell wall, since the blue color (Gram positive) of the wall has changed to red (Gram negative) after the electrolysis. This modification in staining is due to lesion in the cell wall and such lesions allowed leakage of cell material and reduction in the seize of the bacteria. One should remember that Gram staining is dependable on characteristics of the cell wall to fix the complex made between the staining substance (violet crystal) and lugol when exposed to alcohol (LAPERNT; GOURGAUD, 1975). The occurrence of regions where the wall is discontinued or shows alteration allows the alcohol to wash up the complex from the interior of the cell. This explains the reversion of the gram characteristic of the bacteria after treatment.

Cell substances may be precipitated by electroporation (STAPULIONIS, 1999). This electroporation is not prevented in the presence of the cell wall (XUE et al., 1999). Thus, the reduction in size of the *B. subtilis* after electrolytic treatment may be caused by the precipitation of cell substances besides the alterations in the cell wall.

This alteration in the cell wall of the *B. subtilis* may be caused by charge transfer while the microorganism was in contact with the cathode, since the dialysis membrane covered the anode and the environment was chlorate free.

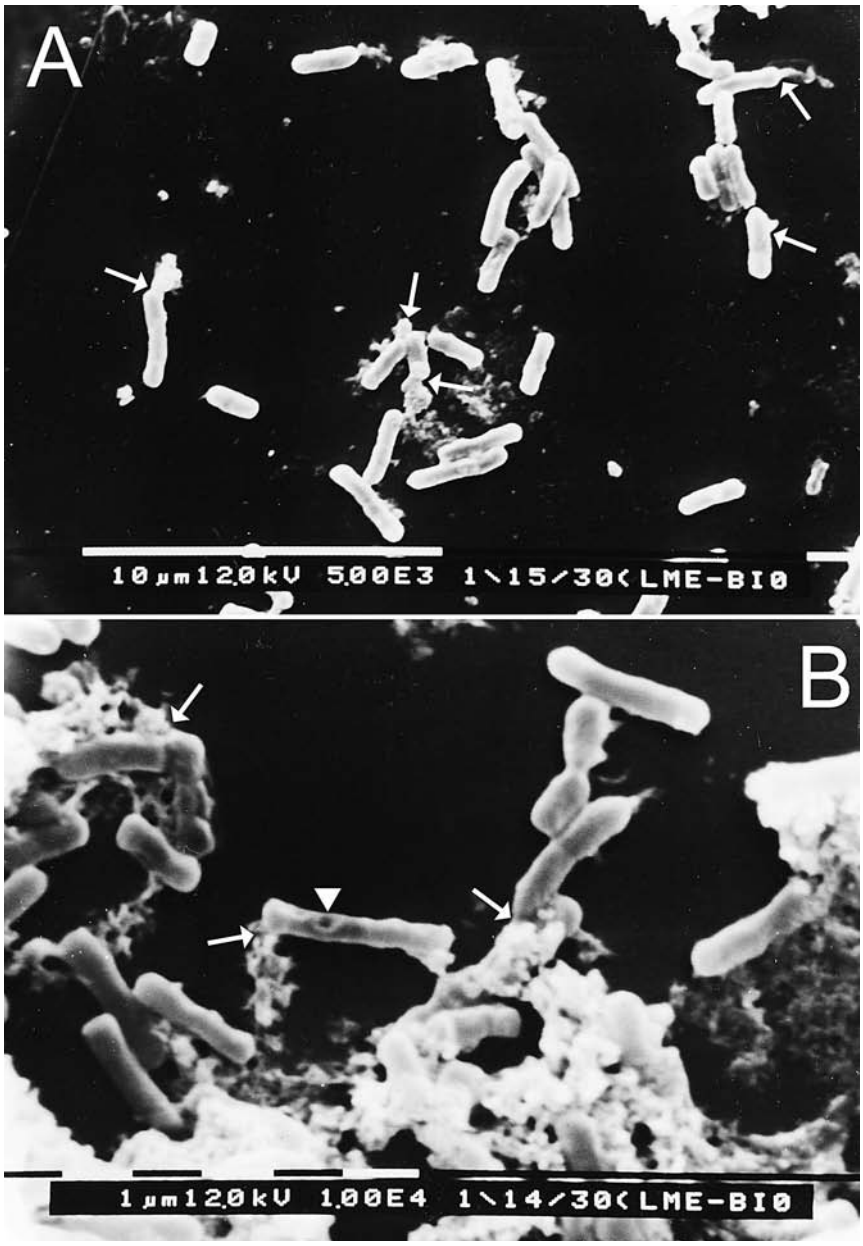
Scanning electronic microscopy (SEM)

FIGURE 2 shows the morphology of *B. subtilis* before electrolytic treatment. Regular rod-like cells can be seen. The surface of the cell wall is regular and shows no lesions.



scale: A =10 µm; B =1 µm.

FIGURE 2: SEM micrograph of the *B. subtilis* before the electrolytic treatment.



scale: A=10 µm; B=1 µm.

FIGURE 3: SEM micrograph of the *B. subtilis* after 30 minutes of electrolytic treatment in buffer phosphate pH 7.2. I = 0.60 A. (arrow) points of leakage of cell material; (m) cell material; (arrow head) rupture of the cell wall.

In non-electrolyzed suspension of *B. subtilis* the bacilli showed a length varying from 1.6 to 4.8 µm and average width of 0.6 µm. In the

micrographs there is no leakage of cell material and the surface of the wall maintains well the rod-like shape.

In FIGURE 3 it is possible to see the morphology of the *B. subtilis* after exposure to 30 minutes of electrolytic treatment. The bacilli have an irregular shape, the cell shows points of leakage of cell material (arrows) and there is rupture of the cell wall and a considerable amount of over-flowed cell material (arrow head).

The surface of the wall seems to be quite irregular if compared to the wall before the treatment. The cell of *B. subtilis* had a length of 1 to 3 µm and the average diameter was 0.4 µm after 30 minutes of electrolytic treatment. Thus, the cell shows a decrease in size after the treatment.

Comparing the results of the observation of *B. subtilis* on SEM before and after the electrolytic treatment it is possible to conclude that the electrolysis caused alteration in the cell wall of the bacteria.

The results from SEM are consistent with those of light microscopy. In both techniques it is possible to observer alterations in seize and shape of the *B. subtilis*. The modification in Gram staining after electrolysis indicates that the bacteria undergo modification in its permeability, which leads to over-flowing of cell material as observed in FIGURE 3.

The increase in cell permeability is generated by electroporation (LUBICKI; JAYARAM, 1996; FRIENDRICH et al., 1998; LEE; TAI, 1999). The potential used during the treatment ranged from 8.7 to 11.9 V, which is enough to induce such phenomenon (LUBICKI; JAYARAM, 1996). Irreversible elecroporation is probably the main cause for killing the *B. subtilis.*

B. subtilis death was not caused by oxidation of coenzyme A, as suggested by Matsunaga et al., 1992 e Okochi et al., 1999, since the oxidation reactions were prevented by isolation of the anode.

The disinfection promoted by the formation of gaseous chloride (Cl_2), or its derivates, did not occur since the system was chlorate free.

CONCLUSION

Results obtained lead to the conclusion that the electrolytic treatment is efficient in the disinfection of water contamined with Gram-positive bacteria such as *Bacillus subtilis*.

The electrolytic treatment promotes alterations in the structure of the cell wall of the *B. subtilis*. There alterations are due to charge transfer in the interface electrode/solution.

The reversion of the bacterial stain by Gram method is due to the modification in the permeability of the cell wall of the *B. subtilis*.

The probable mechanism of disinfection is the association of the effect of elecroporation associated to the transformation due to charge transfer when the microorganism has contact with the electrode.

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To the Laboratory of Electronic Microscopy of the Institute of Biosciences of Rio Claro-UNESP

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