STUDIES REGARDING CHAOTROPIC EFFECTS OF SODIUM PERCLORATE ON *ZYMOMONAS MOBILIS* BACTERIAL MEMBRANE

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Abstract: Chaotropic agents decrease the strength of hydrophobic interactions between nonpolar compounds. The aim of this study is to present the chaotropic effects of sodium perchlorate on bacterial membrane. The membrane permeabilization by chaotropic treatment was highlighted using PAGE electrophoresis and fluorescent microscopy with hypericin from Hypericum perforatum as an effect marker.

Electrophoresis for membrane enzyme activity of ALP, ATP-ase, G6PDH and GFOR revealed perchlorate effect. The two ADH isoforms deserve further attention. This technique can be useful in membrane enzymes extraction in biotechnological purpose. On the other hand, since Z. mobilis is very difficult for genetic manipulation, the use of $NaClO_4$ can be a solution for membrane permeabilization in perspective of genetic engineering.

INTRODUCTION

The plasmatic membrane and the cell wall have the role of protecting the cell from the environment, maintaining within their boundaries the right conditions for metabolic reaction and also ensuring energy and matter transfer between the cell and the environment (Hansruedi, 1981).

The form of the bacterial cell is given by the cell wall. For both Gram positive and Gram negative bacteria, the cell wall contains peptidoglycans (chains of glycans connected to each other through extensible peptides, which protect the cell from lyses because of their elastic structure). The cell wall glycans initially contain up to 100 disaccharide units, with each unit being synthesized so that it connects to a peptide that can later attach to a peptide belonging to another glycan chain (Huang et all, 2008).

Chaotropic agents

Chaotropic agents and ions in particular, have the capacity of loosening the strength of the hydrophobic interactions between apolar compounds by destabilizing the structure formed by water molecules. They have been therefore used in the study of proteins or membranary lipids and enzymatic complexes, by increasing their degree of solubilization.

An impediment in studying particles such as proteins, enzymes, membranary compounds, hem, purines, pyrimidines or some vitamins is their strong hydrophobic reaction (given by their significant number) which makes them highly stable in water.

Chaotropic agents allow proteins to unfold and thus expose their hydrophobic core by changing the structure of the hydrogen bond. In effect, the contact energy between the hydrophobic residues in the solution decreases. One chaotropic agent used despite its low solubility in water is thiourea (Herbert, 1999).

One of the objectives of this study is to demonstrate the applicability of the chaotropic agents treatment on the Z. mobilis bacterial cells as an efficient and profitable method of permeabilization of the cell wall.

The sodium perchlorate chaotropic agent chosen for the experiment was used on the bacterial cells for the first time.

MATERIALS AND METHODS

Sample preparation. For this study were used NCIB 11163 and NCIB 11163/70 *Z. mobilis* strains, the latter being a mutant strain derived from NCIB 11163.

The cell cultures have been grown on a liquid media that contains: 2g% glucose / sucrose, 0.5g% Yeast Extract, 0.5ml% solution $(NH_4)_2SO_4$ (20%), 0.5ml% solution $MgSO_4$ (10%), 1ml%, solution KH2PO4 (10%). (Douka *et all* 1999). The cell cultures were incubated at 30ºC with shaking at 60rpm.

For the experiments developed on cells found in the exponential growth phase incubation was stopped at a rate of absorbance situated between 0.550 and 0.600 and for those that reached the peak of the growth incubation was stopped at a rate of absorbance situated between 1,300 and 1,400.

After stopping incubation 100 ml of liquid culture have been kept on ice for 10 minutes and then centrifuged at 5000 rpm and 4ºC for another 10 minutes. The supernatant was removed and the sediment was resuspended in 1 ml of physiological serum (0.9% NaCl in distilled water), washed through vortex and then centrifuged at 10000 rpm for 10

minutes. The washing operation was repeated for 3 times. After the last centrifugation the resulted sediment was resuspended in 0.5ml of physiological serum.

The resulting samples were subjected to alkaline hydrolysis in NaOH 0.5N for 30 minutes at 60ºC. After this operation the protein was dosed according to Lowry method (Lowry et all., 1951) and the samples were subjected to sodium perchlorate chaotropic agent treatment.

The reaction blend contains 50 mM Tris HCl tampon, 1mM EDTA pH 7.5; 5mM Ditiothreitol (DTT) solution; $NaClO_4$ 0.5M solution; cellular sediment and distilled water. Depending on the protein concentration the total reaction volume was calculated so that it would be uniformized at 10 mg/ml.

The treatment was carried for 30 minutes at 30ºC and 300 rpm shacking speed, after which the samples were centrifuged at 12000 rpm for 10 minutes. From the supernatant 200 µl were set for dialysis in Tris-EDTA tampon for 24 and 90 hours. Over the cellular sediment resulted in the centrifugation Tris-EDTA tampon was added, in an equal volume as the initial volume of the reaction with the chaotropic agent. The samples were then incubated with shacking at 30ºC and 300 rpm for 24 and 90 hours.

At the end of these intervals the samples were centrifuged at 12000 rpm and the supernatant was processed as follows: after 24 hours the protein resulted after dialysis and tampon extraction was dosed through Bradford method (Bradford, 1976). After 90 hours the samples resulted after dialysis and tampon extraction were protein dosed through Lowry method with a Bio-Rad D.C. protein assay kit.

The remaining cellular sediment after the centrifugation of the cells in Tris-EDTA buffer was resuspended in a physiological serum volume equal to the initial volume of the reaction with the chaotropic agent and then sonicated on ice for 6 X 10 seconds.

To reveal the enzymatic activities polyacrylamide gels were used by subjecting the samples to migration on the previous mentioned gels in the presence of Nonidet (undenatured nonionic detergent) at 4ºC.

The concentration of the separation gel was 7.5%, and that of the concentration gels was 4%. The tension induced for the migration process was 70V until the samples went through the separation gel and the increased to 90V.

Glucose 6 phosphate dehydrogenase: after electrophoresis the gels were incubated 15 minutes in Tris-HCl 25mM; $MgCl_2$ 5mM, pH 7,4 buffer. The evidencing system was composed of: 10 ml Tris-HCl, 0,2 M, pH (7,4-8,0); buffer, 1 ml $NADP^+$, (1%); 20 mg Glucose 6 phosphate, 1 ml NBT (1%); 0.5 ml PMS (1%); 25 mg EDTA

Alcohol dehydrogenase (ADH): 0,4 ml ethanol 95%, 5 mg NAD^+, 3 mg Nitro-blue tetrazoliu, 0,2 mg phenazine methosulphate in 9,6 ml Tris-HCl pH 8 buffer**.** (O'Mullan et all, 1994)

Glucose fructose oxidoreductase (GFOR): 0,4 M glucose, 0,8 M fructose, 2 mM NADP+, 3 mg Nitro-blue tetrazolium, 0,2 mg phenazine methosulphate, 20 ml 10 mM MES-KOH pH 6,4 (Stoian e*t. all* 2005)

NADPH dehydrogenase: 3 mg NADPH, 5 mg Nitro-blue tetrazoliu in 50 mM MES-KOH pH 6,4 (Quilles et all).

NADH dehydrogenase: 3 mg NADH, 5 mg Nitro-blue tetrazoliu in 50 mM MES-KOH pH 6,4 (Quilles et all).

Hypericin coloration. For observing the membrane degree of permeabilization by the chaotropic agent an alcohol hypericin solution (1.01×10^{-2} µM concentration) was used. The pictures were taken by an Olympus CX 31 fluorescence microscope, endowed with a 100X Plan C immersion objective.

RESULTS AND DISCUSSIONS

Alcohol dehydrogenase

ADH is involved both in ethanol production and regeneration of NAD^+ in the fermentative metabolic pathway. Z. mobilis posses 2 isoforms named ZADH 1, and ZADH 2. Both are capable to reduce acetaldehyde to ethanol.

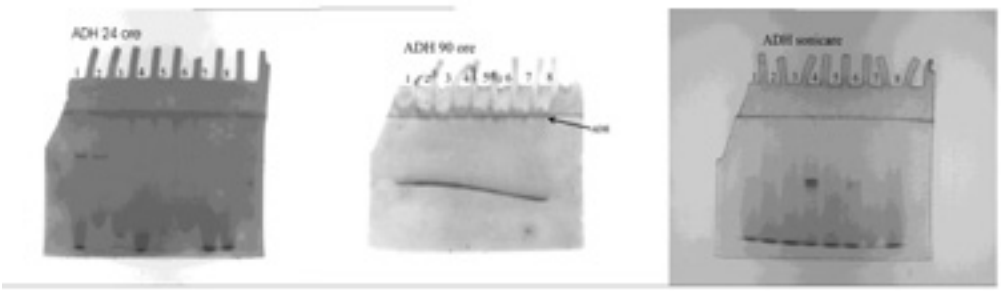

Figure 1. ADH evidentiation. 1-NCIB11163/w.t. sucrose in exponential growth phase, 2-NCIB11163/w.t. sucrose in the peak growth phase, 3-NCIB11163/w.t. glucoze in exponential growth phase, 4-NCIB11163/w.t. glucoze in the peak growth phase

Presence of ADH in 24 and 90 hours buffer proves that the enzyme was easily extracted with the method presented. This indicates a membrane localization of the enzyme, especially ADH 2 isoform witch is a new discovery that could prove important in biotechnology.

Glucose 6 phosphate dehydrogenase

Glucose 6 phosphate dehydrogenase is the enzyme that assures NADPH intracellular regeneration (Ai Hyang Shina, 2004), indirectly contributing to glutathione and oxidized proteins regeneration. Is one of the enzymes implicated in Entner-Doudorof pathway.

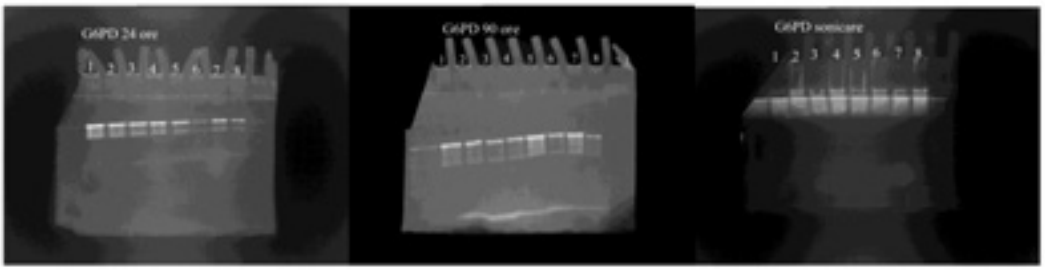

Figura 2. Glucose 6 phosphate dehydrogenase evidentiation: 1-NCIB11163/w.t. sucrose in exponential growth phase, 2-NCIB11163/w.t. sucrose in the peak growth phase, 3-NCIB11163/w.t. glucoze in exponential growth phase, 4-NCIB11163/w.t. glucoze in the peak growth phase

Enzyme activity on all three electrophoretical gels suggests membrane localization of G6PD. The two strips revealed indicate two isoforms.

Glucose fructose oxidoreductase (GFOR)

Periplasmatic localization of GFOR represents an export method of $NADP^+$ to the periplasmatic area of the cell. (Wiegert et all 1996). The way that the $NADP^+$ is exported is not yet known for Z.mobilis (Sprenger,1996; Wiegert et all, 1996).

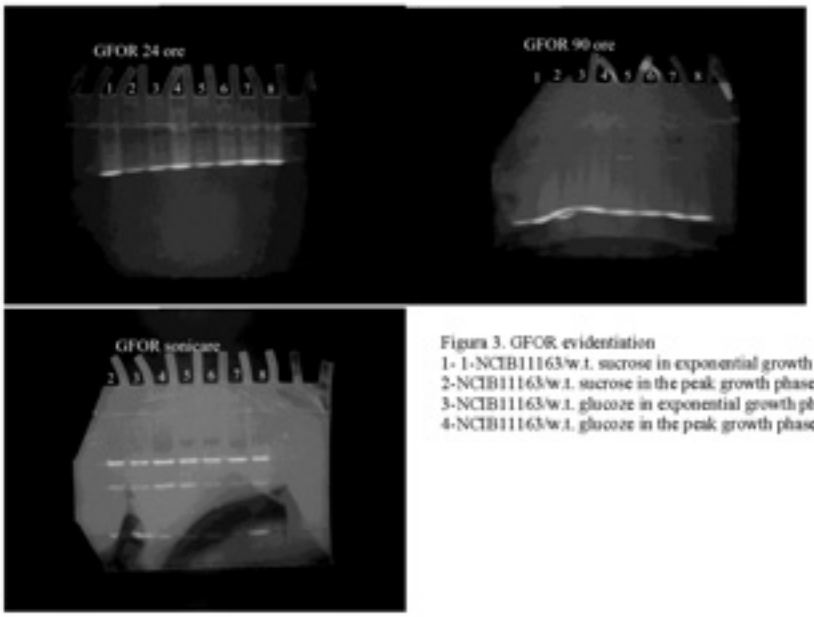

Figura 3. GFOR evidentiation
1- 1-NCIB11163/w.t. sucrose in exponential growth phase
2-NCIB11163/w.t. sucrose in the peak growth phase
3-NCIB11163/w.t. glucoze in exponential growth phase
4-NCIB11163/w.t. glucoze in the peak growth phase

On the sonication loaded gel two isoforms can be found. The chaotropic treatment released only one of these forms.

NADH oxidase

NADH oxidase (EC 1.6.99.3) are enzymes described for aerobic and anaerobic bacteria. Their role is to mediate direct electron transfer from NADH and molecular oxygen. These enzymes allow bacteria to use environmental oxygen (Thad and colab, 1993).

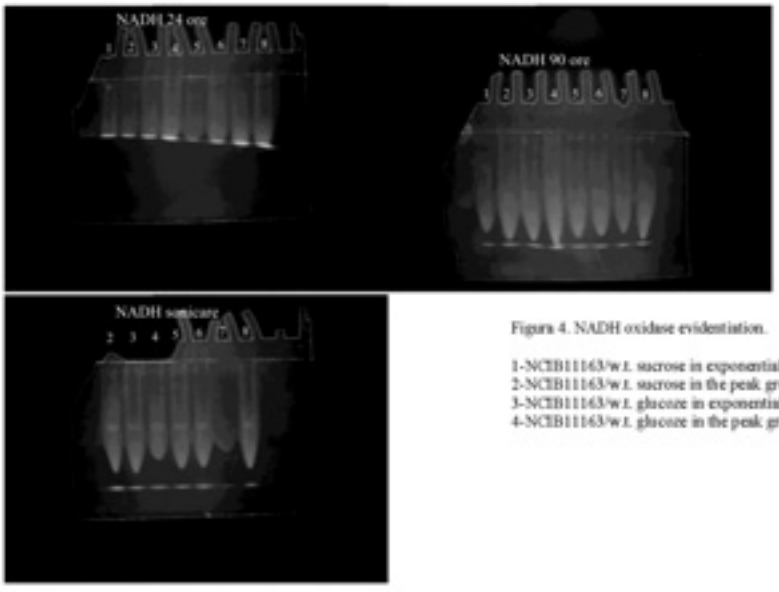

Figura 4. NADH oxidase evidentiation.

1-NCIB11163/w.t. sucrose in exponential growth phase
2-NCIB11163/w.t. sucrose in the peak growth phase
3-NCIB11163/w.t. glucoze in exponential growth phase
4-NCIB11163/w.t. glucoze in the peak growth phase

In this case the chaotropic treatment did not had any effect, enzyme activity is visible only on the sonication loaded gel.

NADPH oxidase

NADPH oxidase activity in Z. mobilis presents similarities with citocrom b oxidase found in NADPH membrane oxidase system from mammal fagocites (Fujii et all, 1995). Other flavoproteins like 77-kDa protein (Laporte et all, 1991) and nitro blue tetrazolium reductase (Miki et all, 1992), have been reported as candidates for NADPH dehydrogenase activity.

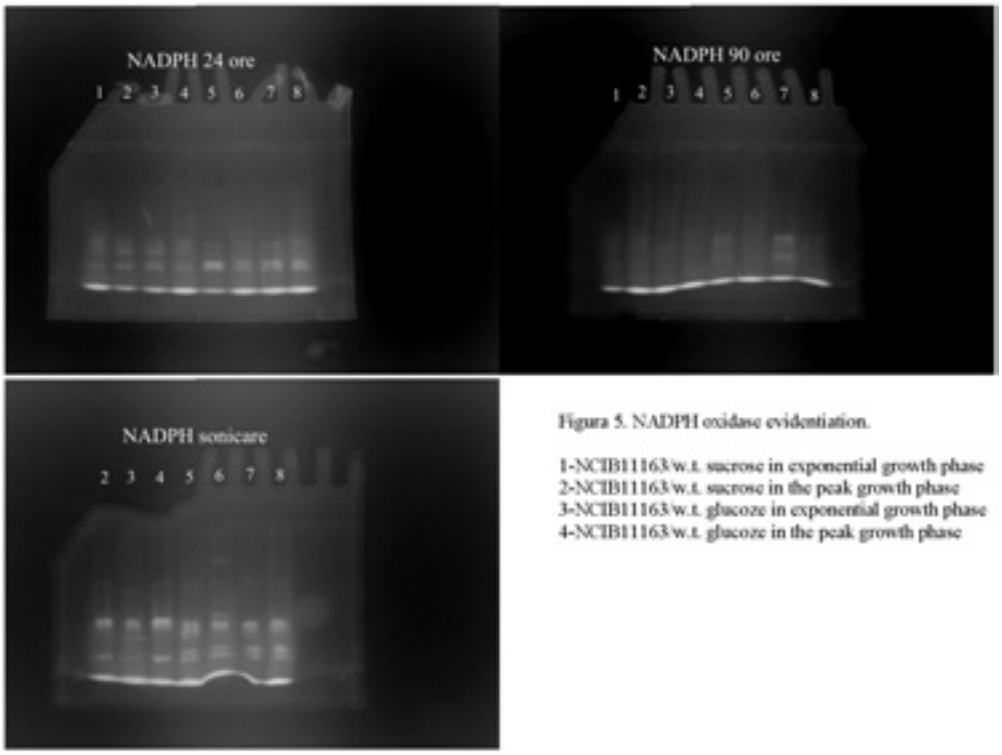

Figura 5. NADPH oxidase evidentiation.

1-NCIB11163/w.t. sucrose in exponential growth phase
2-NCIB11163/w.t. sucrose in the peak growth phase
3-NCIB11163/w.t. glucoze in exponential growth phase
4-NCIB11163/w.t. glucoze in the peak growth phase

In this case the optimum time for buffer extraction was 24 hours. The intensity of the strips on the 24 hours buffer samples loaded gel were similar to the sonication samples loaded gel.

In addition to the electrophoresis the membrane degree of permeabilization was observed in the microscopy part of the experiment. A ethanol solution of hypericin extracted from Hypericum perforatum was used to color the cells. Hypericin penetrates the outer membrane degraded by $NaClO_4$ and reaches in the periplasmatic space from were under UV light it makes the cell “glow”

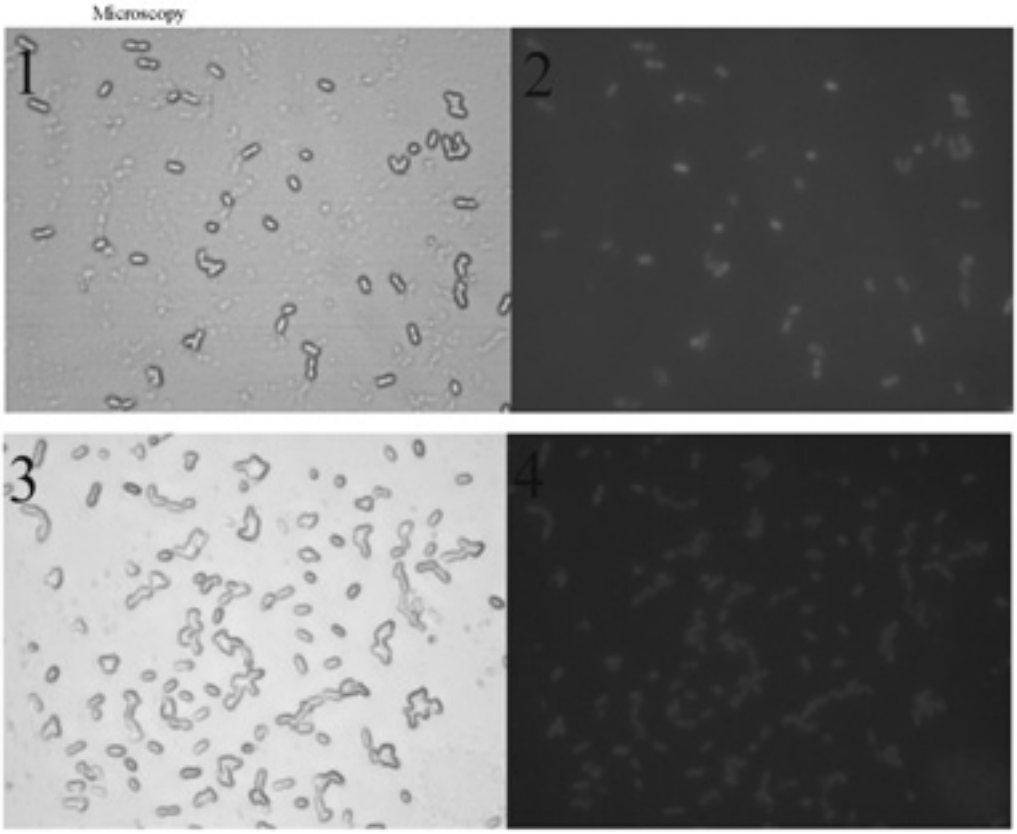

Figure 6. NCIB 11163 strain on glucose medium, exponential growth phase. Legend: 1- cells before treatment; 2- cells before treatment UV; 3-cells after treatment; 4- cells after treatment UV

In visible light it can be observed how hypericin surrounds the treated cells.

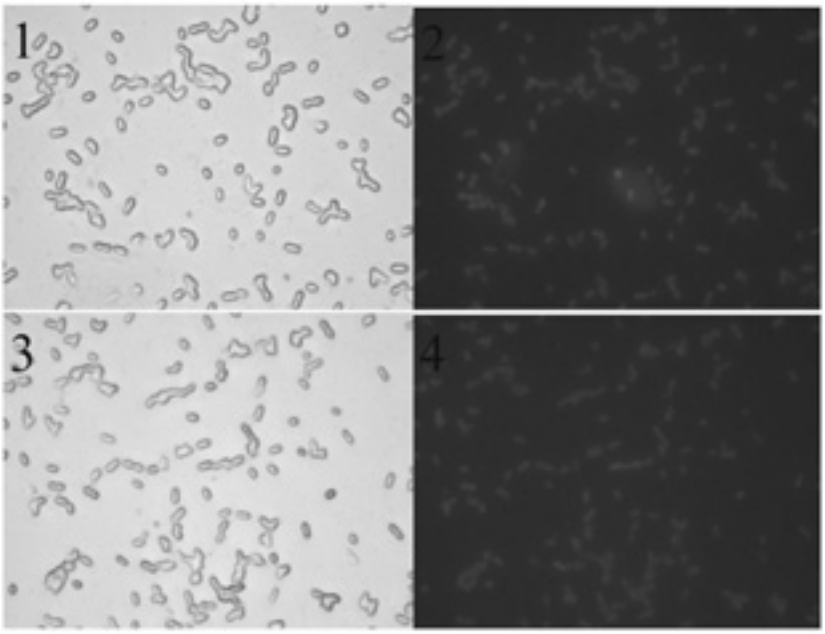

Figure 7. NCIB 11163 strain on glucose medium peak growth phase. Legend: 1-cells before treatment; 2- cells before treatment UV; 3-cells after treatment; 4- cells after treatment UV

After the treatment the cells retain their shape but they are not as well outlined as before.

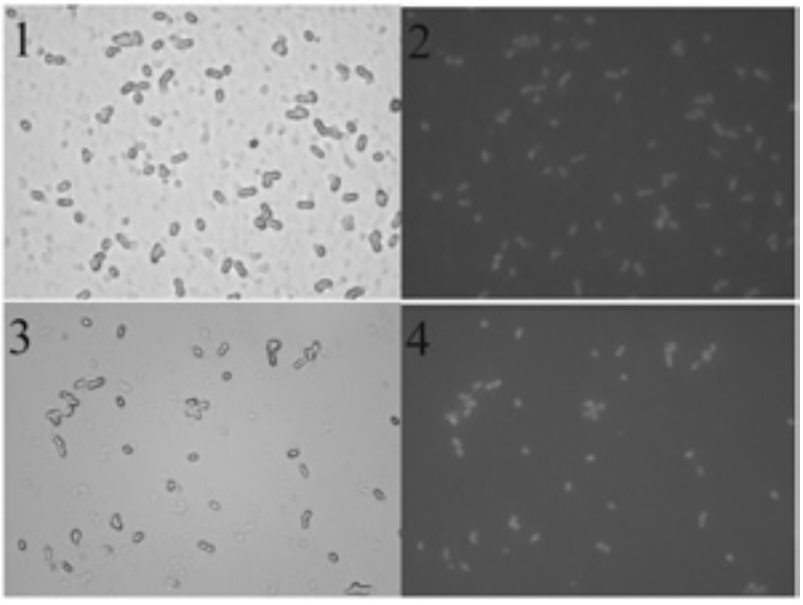

Figure 8. NCIB 11163 strain on sucrose, exponential growth phase. Legend: 1-cells before treatment; 2- cells before treatment UV; 3-cells after treatment; 4- cells after treatment UV

In UV light is clear how hypericin penetrates the cells treated with chaotropic agent, „lightening" the entire cell. This fact proves the permeabilization of the cell envelope by sodium perchlorate

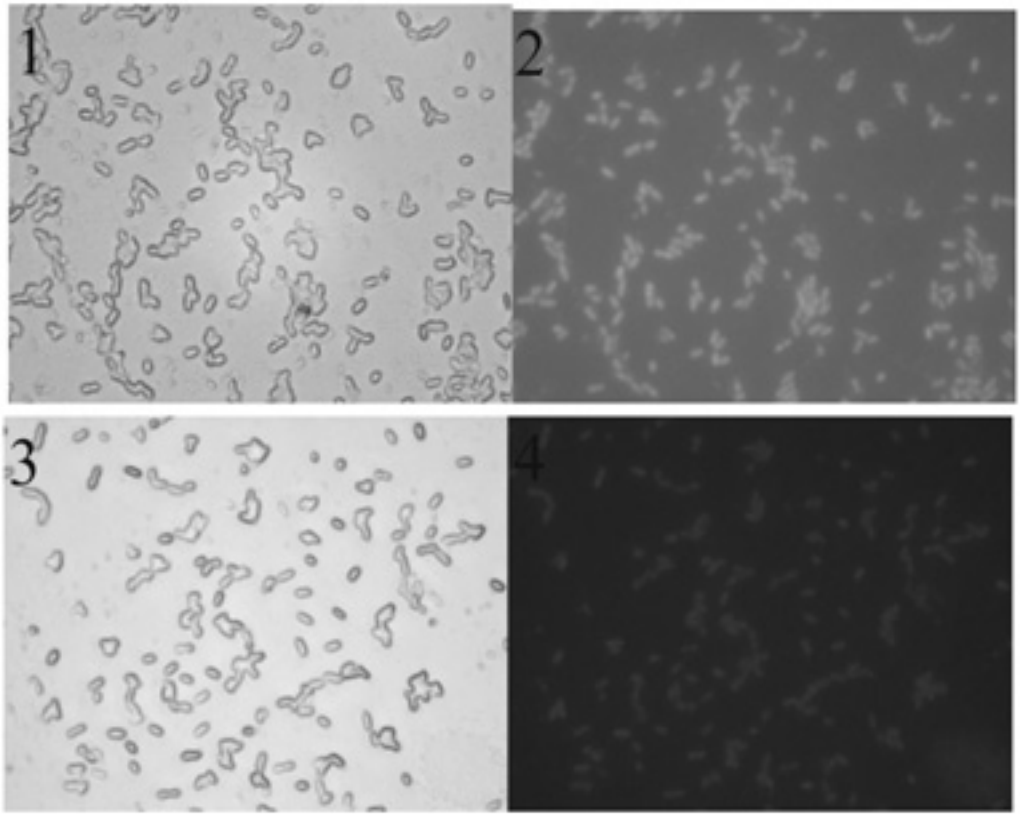

Figure 9. NCIB 11163 strain on sucrose, peak growth phase. Legend: 1-cells before treatment; 2- cells before treatment UV; 3-cells after treatment; 4- cells after treatment UV

Hypericin is very effective as permeabilization marker – 1,01 $x10^{-2}$ µM was enough to visualize the permeabilization process.

CONCLUSIONS

Sodium perchlorate proved it self to be an effective chaotropic agent for Z. mobilis.

The pairs of strips on the electrophoresis gels for ADH, G6PD, and GFOR indicate isoforms of these enzymes worthy to be studied in the future.

Because Zymomonas mobilis is a bacterium difficult to genetically manipulate, $NaClO_4$ treatment is an affordable method to permeabilize the membrane in the perspective of genetically engineering.

Hypericin can be used as a membrane permeabilization marker.

The membrane attached enzymes can be used in biotechnological processes.

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