The Influence of Galvanic Field on *Saccharomyces cerevisiae* in Grape Must Fermentation

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Summary

In *Saccharomyces cerevisiae* alcohol fermentation of Sauvignon blanc grape must a low direct electric current (DC) of 1.3, 7.7 and 30 µA was applied. Constant current stimulated wine yeasts metabolic activity by increasing production of glycerol and lactic acid. The results of high performance liquid chromatography (HPLC) and gas chromatography (GC) indicate that by using the direct current at low temperature, similar results as those using higher fermentation temperatures can be achieved. Optical and transmission electron microscopy showed no visible morphological and ultra structural in cell morphology. The empirical experience resulting from present laboratory experiments enable offer a new approach in fermentation of grape musts wine and in wine process control.

K e y w o r d s : *Saccharomyces cerevisiae*; Wine ; Alcohol fermentation ; Electrostimulation ; Glycerol

Introduction

Introduction of various metal connectors and different metal parts in the construction of fermentors, could induce generation of low current galvanic fields in many fermentation processes. The effects of galvanic fields on microbial physiology could be studied by initiation of AC or DC electrical current by electrostimulation with different pulse amplitudes.

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The effects of electrostimulation on living cells could induce changes in DNA synthesis, protein synthesis, membrane permeability and in cell growth (AMERINE *et al.* 1967).

Although the potential for practical application of electrostimulation to microbial processes is high, investigations dealing with electrical control of microbial fermentation systems, under practical cultivation conditions, are just a few (AMERINE *et al.* 1967, SEI-EOK *et al.*1998).

JACOB *et.al* 1981, studying the influence of static galvanic field on growth of *Saccharomyces cerevisiae* found that the response of microbial growth to electrostimulation in the exponential growth phase was much more expressed than in the stationary phase. This foundings were confirmed also by HÜLSHEGER *et.al* 1983.
Sei-Eok Yun et.al 1998, applied an electric field of 10, 20 and 30 V/cm at frequency of 50 Hz for *in vitro* electrostimulation of suspension of yeast alcohol dehydrogenase. Various changes in promoting or inhibiting enzyme kinetics were observed by several authors (SEI-EOK *et al.*1998), MORTENSEN and BOJSEN 1982).
KARBA *et.al* 1991, investigated the influence of low current static galvanic fields on *Candida albicans in - vitro.* It was found that microbial growth was inhibited in direct proportion to the intensity of the electric strength and its application time. ARONSSON *et al.* 2001, studied the influence of electrical strength and the frequency of the pulsing on growth *Saccharomyces cerevisiae.* 4.7 kV/cm was found as the critical death value for the growth of *S. cerevisia* . In electrostimulation of *Yersinia enterocolitica* ALVAREZ *et.al* 2003 found that increasing of the time of electrostimulation inhibited microbial growth rate.
NAKANISHI *et.al* 1998, measured the effect of electric currents on the stimulation of yeast cell growth including production of alcohol, organic acids and aromatic compounds. 10 mA (DC) and 100mA (AC) were referred to induce significant increases in cell growth and alcohol production rates. Microcurrents could also stimulate production of ATP as a factor of cell energy need for normal function of the cell. Local differences in the ionic area that resulted out of electrostimulation are usually followed by significant changes in membrane potential (ARONSSON *et al.* 2001, ALVAREZ *et al.* 2003). Electrostimulation could also decrease the membrane permeability thus inhibiting the transport ability of the membrane proteins and chanels (WEI CHEN *et al.* 1998, COSTER and CHILCOTT 1999).

Materials and Methods

M i c r o o r g a n i s m : *Saccharomyces cerevisiae* yeast (Fermicru, AR2Val de Loire, France) 2g dry wgt. was reactivacted for 20 min at 30 ºC in a water diluted (1:1 v/v) Sauvignon blanc must. 20 ml of yeast **s**uspension in concentration 10^7 cells/ml was used in all experiments.

F e r m e n t a t i on s u b s t r a t e : Grape juice of Sauvignon blanc, from wine-growing region Ljutomersko-Ormoške gorice, was used as a fermentation media in all experiments. The musts, fermented on the laboratory scale, were not sulphurized before the beginning of the fermentation.

E l e c t r o s t i m u l a t i o n : Constant (DC) electric current of different strength (1.3, 7.7, 30 μA) was applied. Pt /Ir electrodes and agar bridges were applied.

F e r m e n t o r : 10 l stirred tank reactor of standard configuration (Bioengineering AG, Switzerland) was used. It was equipped with reflux cooler column, Ingold pH and redox electrodes, temperature control unit and were stirred at 100 rpm.. For *on-line* measurements, SHIVA control software (BIA d.o.o., Slovenia) was applied. The fementors' head space was filled with N_2 to prevent oxidation of the fermenting grape must.

A n a l y t i c a l m e t h o d s : Organic acids, reductive sugars and alcohol in wine and grape must were analysed by HPLC. Standard validation methods proposed by BIO-RAD 1997, were applied. Measurements of the concentrations of reductive sugar, ethanol, glycerol, concentrations of some organic acids and biomass concentration were *off-line* daily measured. Samples were filtered through a 0,45 μm membrane and analysed using 300 mm × 7,8 mm Aminex HPX-87H organic acid analysis cationic exchange column. Elution was performed at 65 ºC. The mobile phase was 0,005M H_2SO_4 in bi-distilled water. The pump was operating at a flow rate of 0,5 ml/min ($0{,}008 \cdot 10^{-3}$ l/s). The injection volume was 20 μl. The eluting compounds were monitored at 210 nm by a fixed ultraviolet (UV-VIS) wavelength detector. This detector was connected in series with a refractive index (RI) detector. Tartaric and malic acids were detected by UV; citric, succinic acids, glucose, fructose, glycerol and ethanol were detected by RI detector. The peaks were quantified using external standard calibration.

The components were identified by a comparison of their retention times with those of the standards. Quantification was performed using external standards prepared from pure compounds.
Biomass was determined gravimetrically after 5 min centrifugation at 4000 rpm and 24 h drying at 105 ºC. 20 ml of fermentation broth was used.

Light and electron microscopy : Cell morphology was described by staining yeast cells with vital dyes neutral red and methylen blue and examining with light microscope Axioscope, Zeiss. Yeast cells were prepared for ultrastructural analysis by conventional method of fixation in mixture of 1.5 % glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer, postfixation in 1% osmium tetroxide, dehydration in graded series of ethanols and embedding in Spurr. Ultrathin sections were stained in uranyl acetate and lead citrate and examined in a transmission electron microscope Philips CM 100.

Results and discussion

Influence of electrostimulation on growth and metabolic activity of yeast cells was monitored over *on-line* redox potential measurements. Differences between *on-line* redox potential measurements and the control samples were observed. Redox potential in the first aerobic phase in the presence of low oxygen pressure in fermentation must shows small incerasing of redox potential from starting 380 mV to 400 mV. This increase is the most evident at 18 ºC, while at 26 ºC this phase is the shortest. After this phase yeast is turning to anaerobic phase and to production of ethanol (KUKEC *et al.* 2003). In this phase redox potential drops to negative values, - 170 mV at 120 hours of fermentation (18 ºC), - 185 mV at 72 hours (22 ºC) and – 220 mV at 48 hours (26 ºC). Comparing redox potential measuremens of the control samples to the measurements with electrostimulation faster aerobic and anaerobic phases, similar to the control experiment at 26 ºC, but and more intensive microbial activities were observed. All of the changes in redox potential occures already at 38 hours, therefore at 1.3 μA it drops to the final – 300 mV, at 7.7 μA to – 395 mV and at 30 μA to – 420 mV (Figs. 1 a,b).
Biomass production was recognized to be the highest in the samples with electrostimulation.

After 10 days at the end of fermentation at 1.3 µA 4.5 g/l, at 7.7 µA 5.5 g/l, and 6,2 g/l at 30 µA. (Fig. 2).

In the next measurements the effects of electrostimulaton on substrate consumption were studied. In all of the three cases where galvanic field was applied and also in the control experiment, the consumptions of glucose and fructose were nearly equal and similar to the control sample at 26 ºC. Consumption at fermentation temperature 18 ºC and 22 ºC was slowlier and rest of fructose 16.6 g/l and 11.6 g/l was detected (Fig.3 a,b).

Acetaldehyde production reached at non stimulated experiments at the temperatures of 18 ºC and 22 ºC in average 16 mg/l in 4 days while at 26 ºC, 20 mg/l in 60 hours. In the same period in electrostimulated samples acetaldehyde production highly increased to 28 mg/l (1.3 µA), 31 mg/l (7.7 µA) and and 33 mg/l (30 µA). In all these experiments, conversion of acetaldehyde to ethanol was also much faster. It was mostly converted to ethanol in 5 days, while at lower temperature (18 and 22 ºC) control samples this process was slower and it lasted up to 10 days (Fig.4).

Ethanol production, in all of the control experiments was as it follows: after 10 days of fermentation at 18 ºC it was produced 60 g/l, 78 g/l at 22 ºC happen after 8 days and 80 g/l in control at 26 ºC happen in 5 days. With electrostimulation at low current of 1.3 µA ethanol production in 4 days was 85 g/l, while at higher currents of 7.7 µA it was 95 g/l and at 30 µA 99 g/l (Fig.5a). Very similar results were obtained for glycerol production. The application of galvanic fields of different strength yielded a higher and faster glycerol production. Applying the currents of 1.3 and 7.7 µA resulted in 9 g/l of glycerol production in 6 days. At higher current 30 µA in the same time 10 g/l of glycerol was detected.
(Fig.5 b).
Metabolic activity of wine yeast was also controlled by measuring of isoamyl alcohol, 1-propanol and 2- butanol accumulation. While at 26 ºC in control as well as in low current field of 1.3 µA, 75 g/l of isoamyl alcohol was obtained at higher currents, at 7.7 and 30 µA 85 g/l were produced in 5 days. At higher currents of 7.7 and 30 µA also higher amounts of 1-propanol 45 g/l and of 2- butanol 49 mg/l were obtained in 5.5 days (Figs 6 a,b,c).

In measurements of organic acids the amount of tartaric acid (5.8 g/l at final) was nearly unchanged in all the cases of electrostimulation while its conversion rate in comparison with control was much faster. Also malolactic conversion to lactic acid, proceeded in 5.5 days up

to 4.4 g/l in all experiments where galvanic field was applied. 50 % higher yield was produced (Figs.7 a,b,c).

The cell shape and the ultrastructure of electro-stimulated yeast cells did not change compared to the control cells. Ultrastructural analysis showed that the fibrillar cell wall is composed of two layers, periplasm is electron lucent and cell membrane forms invaginations. Nucleus is surrounded by cellular inclusions, mostly lipid droplets and vacuoles, single mitochondria are present (Figs. 8 a,b).

Conclusions

Bioelectrical current dynamics represents one of the most relevant characteristics of biological systems that are playing a significant role in differentiation, growth, tissue and microbial cell regeneration. Various biological liquids consist of a large amount of water, ions, polare molecules, charged proteins, lipids, hormons and coloid particles. The most relevant carriers of electrical currents are various ions. At low current densities their electrical conductivity is linear while at high current densities various nonlinearities are more evident (OMORI *et al.*1996).

Galvanic fields could also influence changes in the cell membrane layers at membrane surface structures inducing relevant changes in selective transport of ions or polar molecules through the cell membrane. These changes could significantly affect functioning of cell metabolism and the changes in cell organelles promoting or diminishing cell metabolism (OMORI *et al.*1997, BARNES 1985).

The cell shape and the ultrastructure of electrostimulated cells did not differ significantly from that of non-stimulated cells. The fibrillar structure of the cell wall was preserved, the periplasm was electron-lucent, nucleus was surrounded by vacuoles and lipid droplets. Cell membrane was deeply invaginated at certain areas which could be related to increased kinetics in electrostimulated cells.

In kinetics of wine yeast metabolites formation direct electric current (DC), of 7.7 and 30 μA, was found as a promoting factor that stimulate faster and more intensive product yielding cell metabolism. Application of electro-stimulation in grape must alcohol fermentation at 18 ºC showed an increase in the rate and, as in the most of the cases, in higher amounts of the fermentation products. The results where electro-stimulation of the wine yeast cells was

applied are comparable to those where fermentation proceeded at temperature of 25 ºC. Besides in the case of tartaric acid conversion, application of a low current galvanic field showed, that application of direct electric current of 7.7 and 30 µA, significantly affected stimulation of faster and higher production of alcohols and organic acids. Between this products the most remarkable increase belongs to the production of lactic acid and glycerol, the most relevant compound that contributes to smoother and more complete complexity of the wine. Presented results are a new approach in the knowledge of grape musts alcohol fermentation that could significantly contribute to the quality of wine and its process control.

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Figures

Fig. 1 a,b Fermentation *on-line* redox potential courses.
1a : ♦T = 18, ■ 22 and ▲ 26 °C , without electrostimulation
1 b : ◊ T = 26° C (1,3 μA) , □ T = 26° C (7,7 μA), Δ T = 26° C (30 μA), with electrostimulation.

Fig. 2 Biomass production.
♦ T = 18° C , ■T = 22° C , ▲T = 26° C, without electrostimulation ;
◊ T = 26° C (1,3 μA) , □ T = 26° C (7,7 μA), ΔT = 26° C (30 μA), with electrostimulation.

Fig. 3 a,b **a.** glucose consumption, **b.** fructose consumption.
♦ T = 18° C , ■T = 22° C , ▲T = 26° C, without electrostimulation
◊ T = 26° C (1,3 μA) , □ T = 26° C (7,7 μA), ΔT = 26° C (30 μA), with electrostimulation.

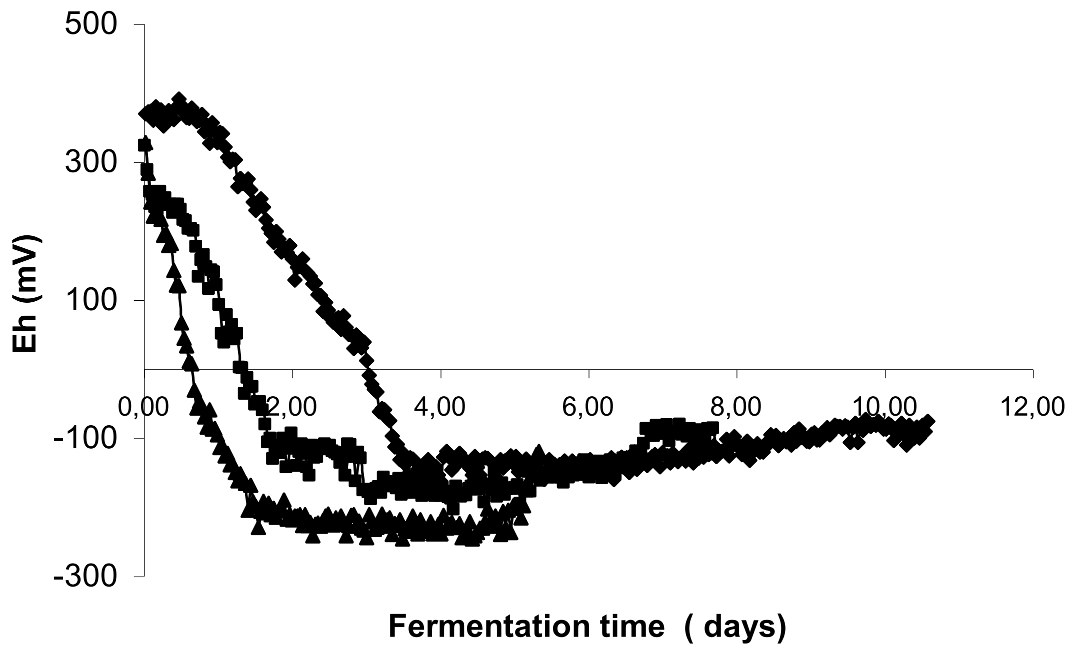
Fig. 4 Acetaldehyde in fermentation time course.
♦ T = 18° C, ■T = 22° C, ▲T = 26° C, without electrostimulation
◊ T = 26° C (1,3 μA) , □ T = 26° C (7,7 μA), Δ T = 26° C (30 μA), with electrostimulation.

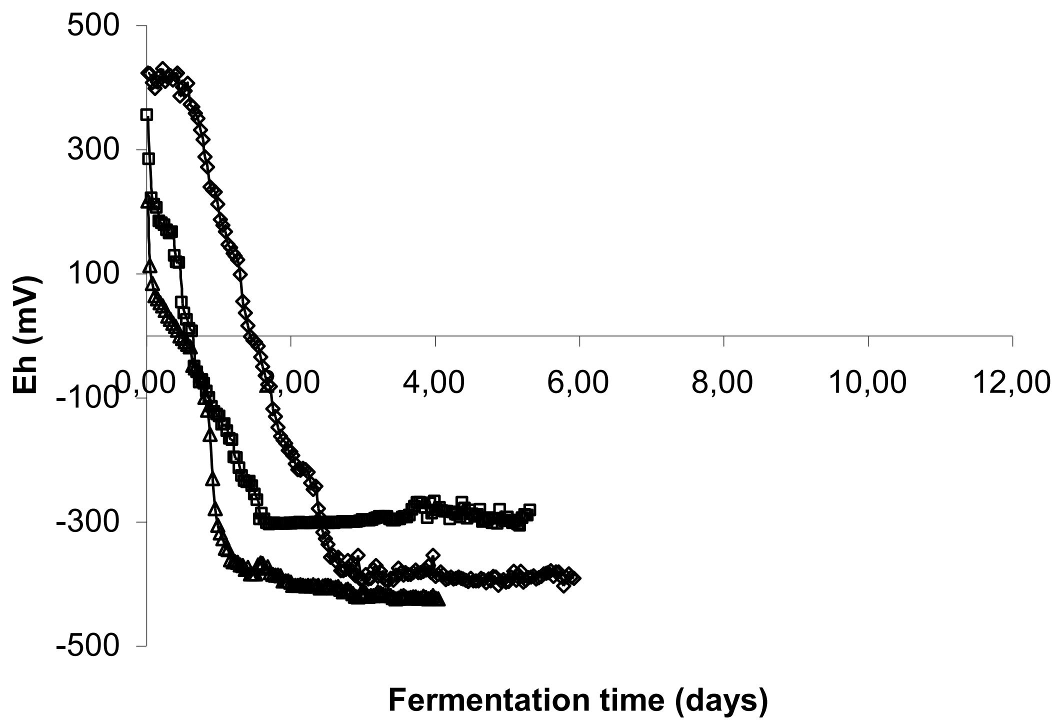
Fig.5 a,b **a.** production of ethanol , **b.** production of glycerol
♦ T = 18° C , ■T = 22° C , ▲T = 26° C, without electrostimulation
◊ T = 26° C (1,3 μA) , □ T = 26° C (7,7 μA), ΔT = 26° C (30 μA), with electrostimulation.

Fig. 6 a,b,c **a.** accumulation of iso-amyl alcohol, **b.** accumulation of 1-propanol
c. accumulation of 2-butanol
♦ T = 18° C , ■T = 22° C , ▲T = 26° C, without electrostimulation
◊ T = 26° C (1,3 μA) , □ T = 26° C (7,7 μA), ΔT = 26° C (30 μA), with electrostimulation.

Fig. 7 a,b,c **a.** Tartaric acid time course, **b.** malic acid time course,
c. accumulation of lactic acid
♦ T = 18° C , ■T = 22° C , ▲T = 26° C, without electrostimulation
◊ T = 26° C (1,3 μA) , □ T = 26° C (7,7 μA), ΔT = 26° C (30 μA), with electrostimulation.

Figs.8 a,b Electro stimulated cells. Chemically fixed and Spurr embedded ultrathin section of yeast suspension in 1% agar. The cell wall shows a fibrillar structure, periplasm is electron lucent, cell membrane is deeply invaginated. Nucleus is surrounded by lipid droplets.





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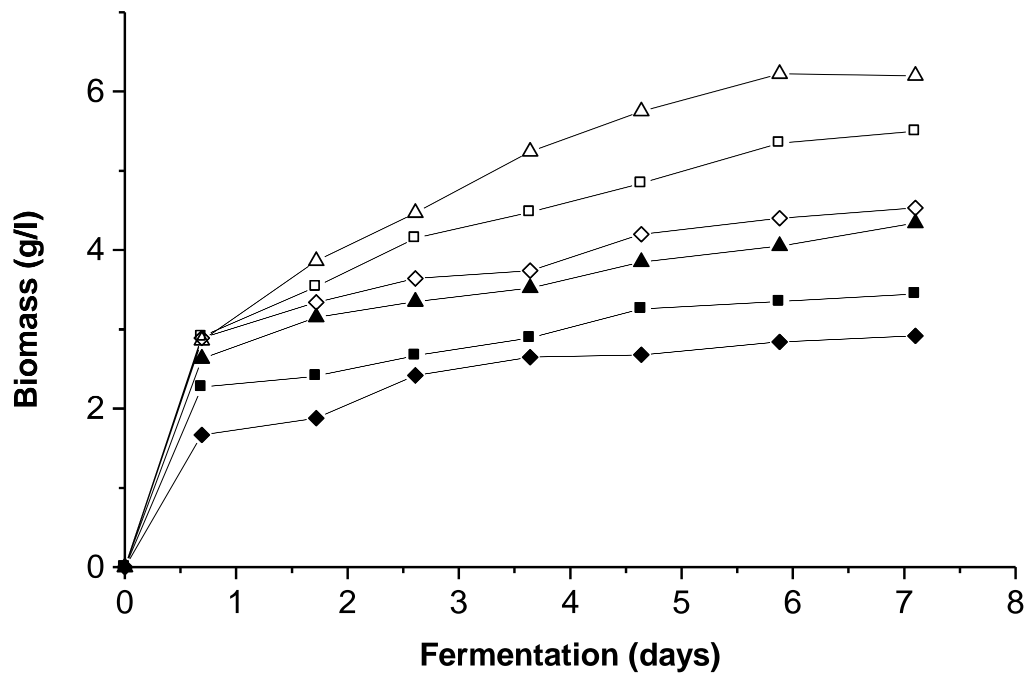

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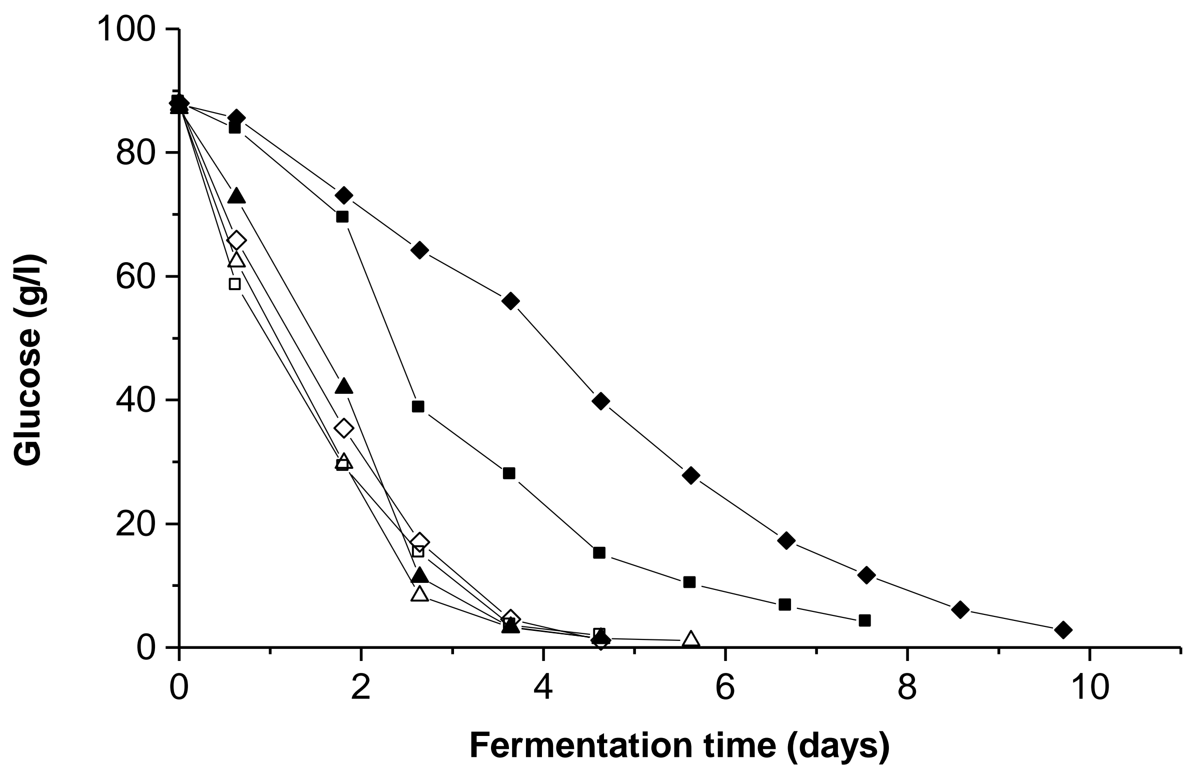

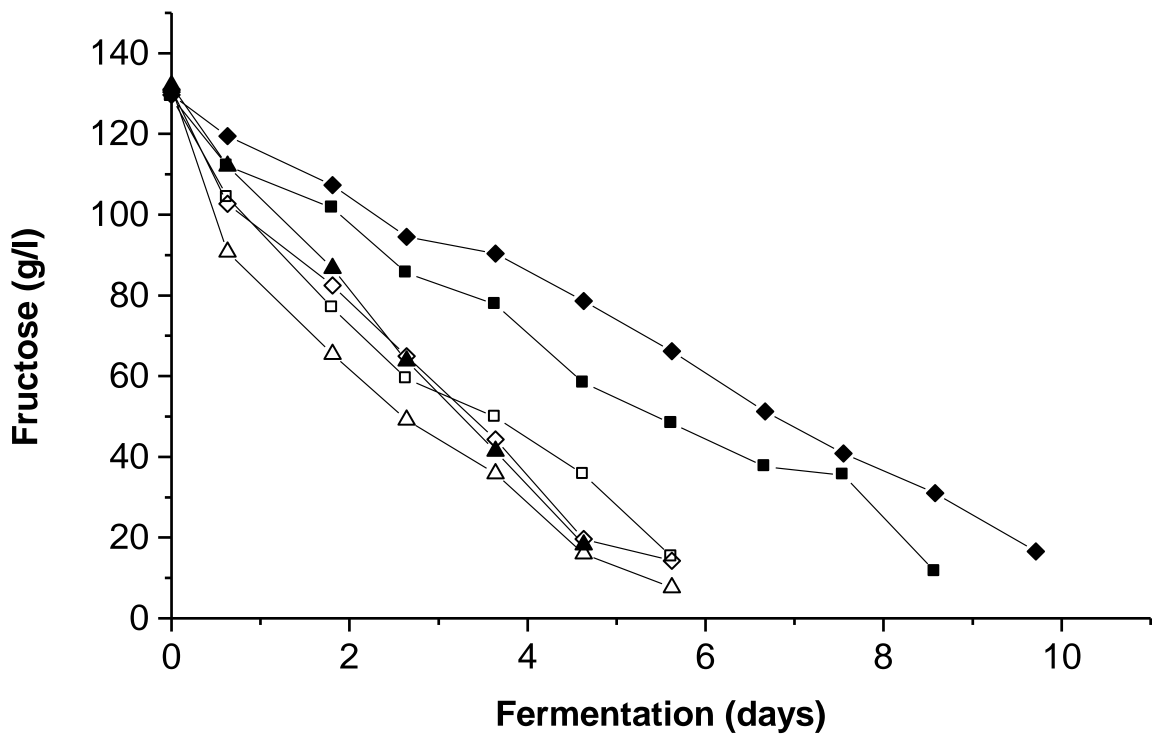

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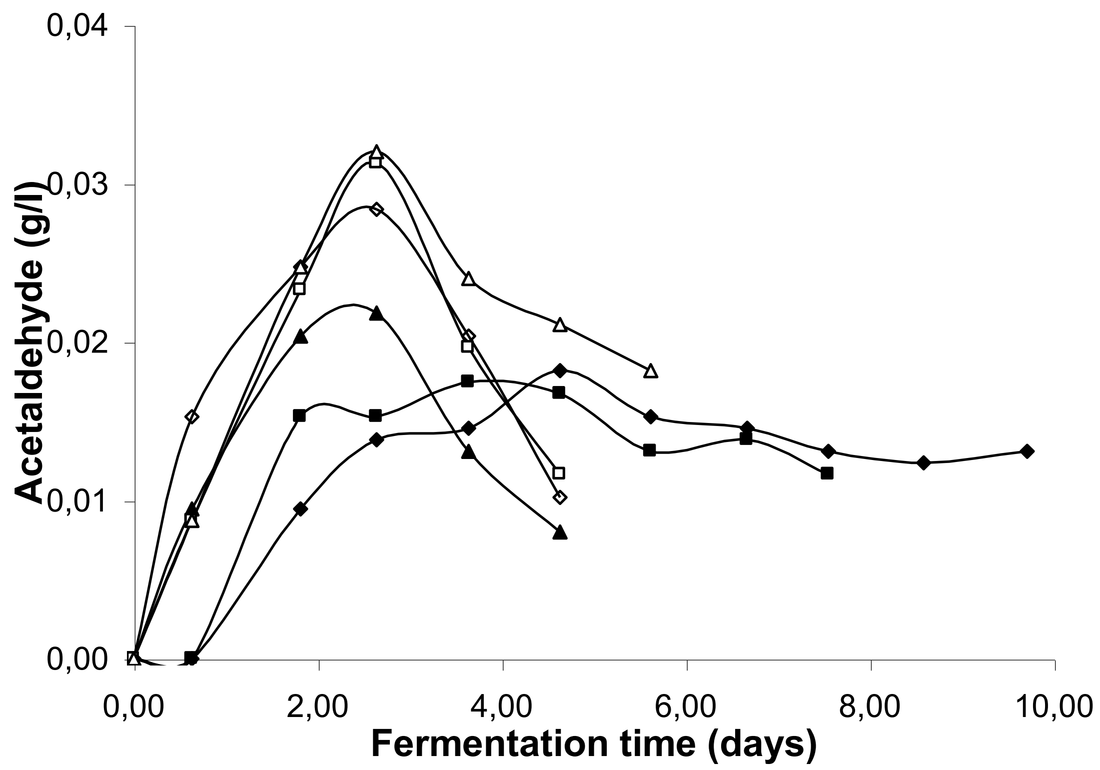

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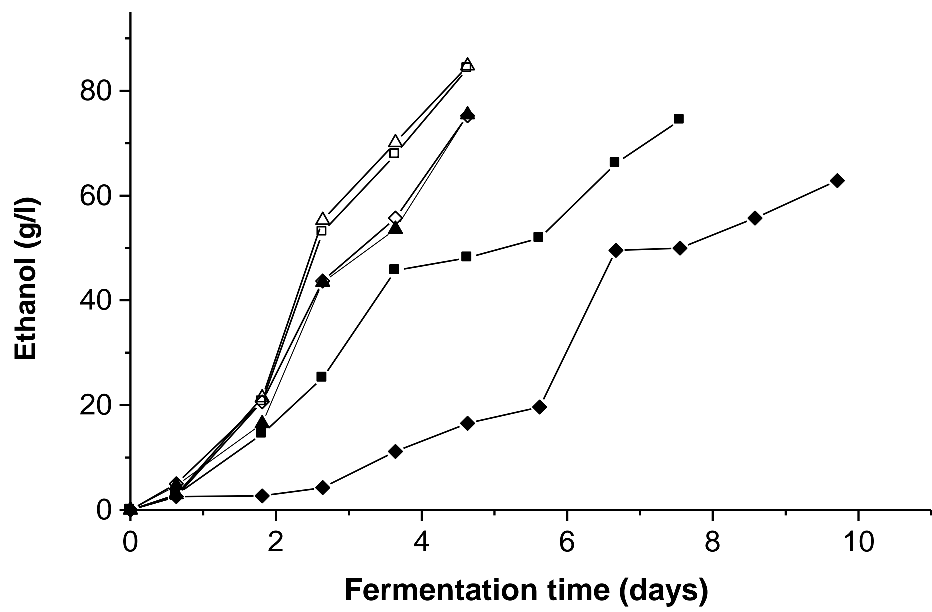

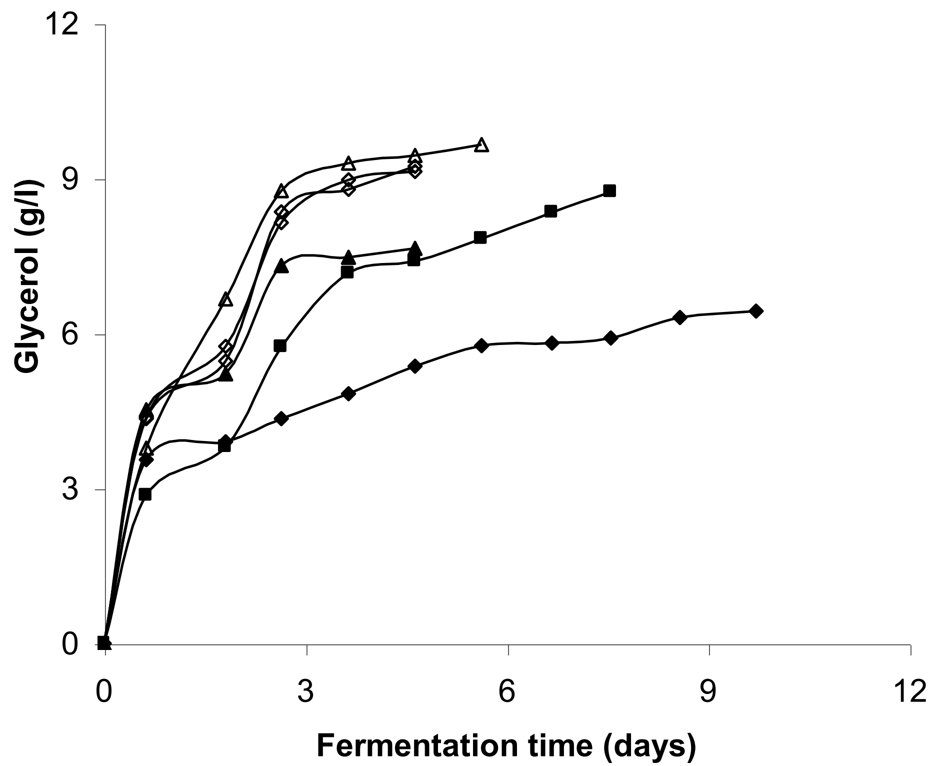

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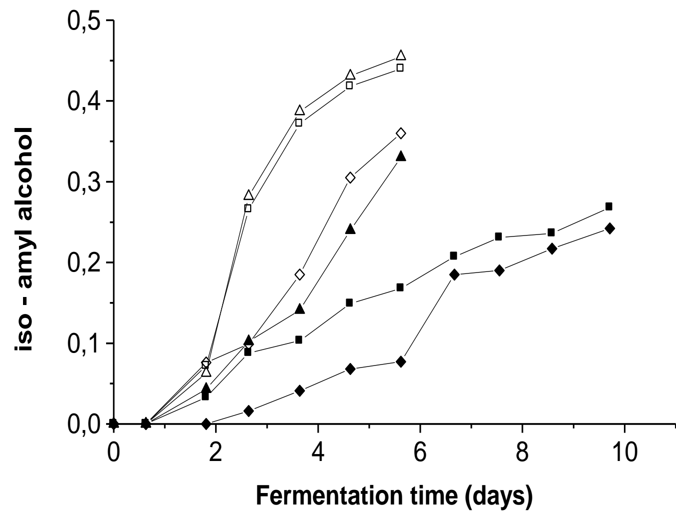
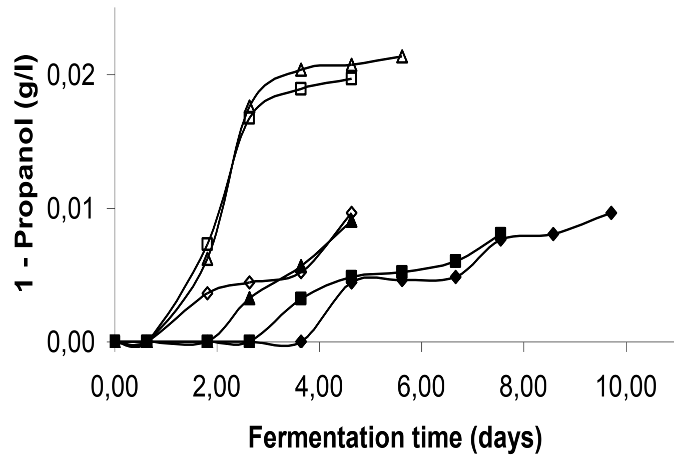




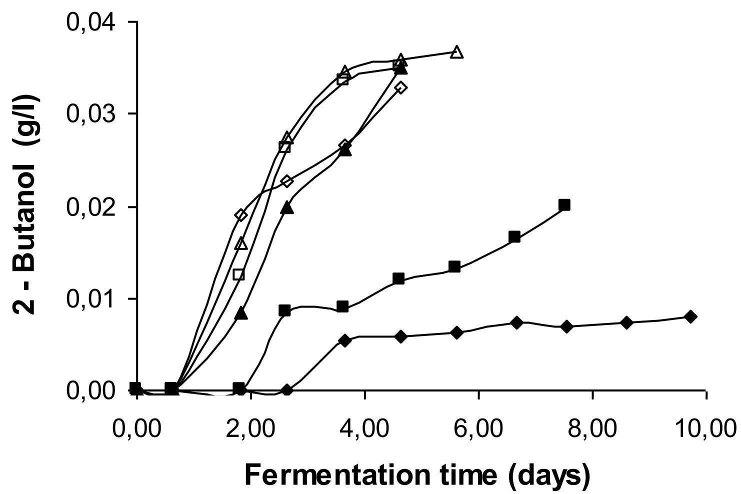
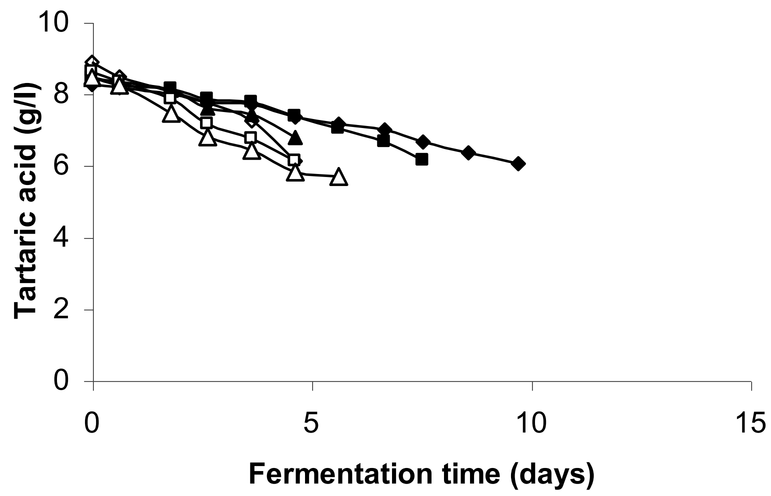
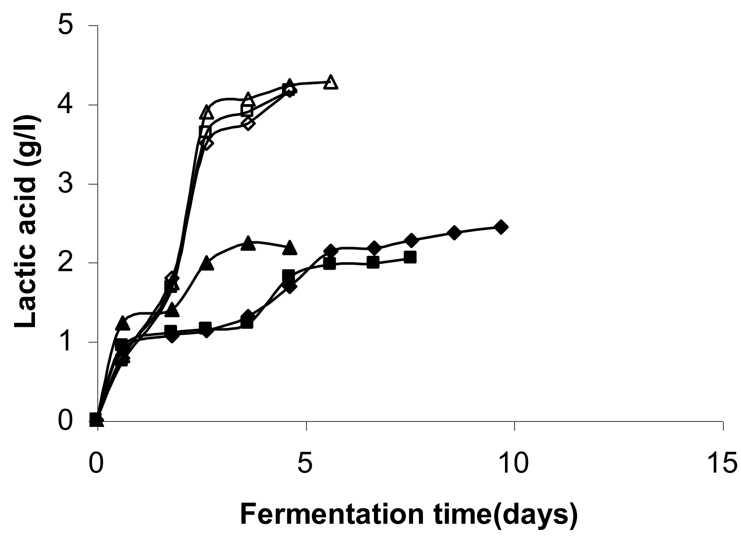
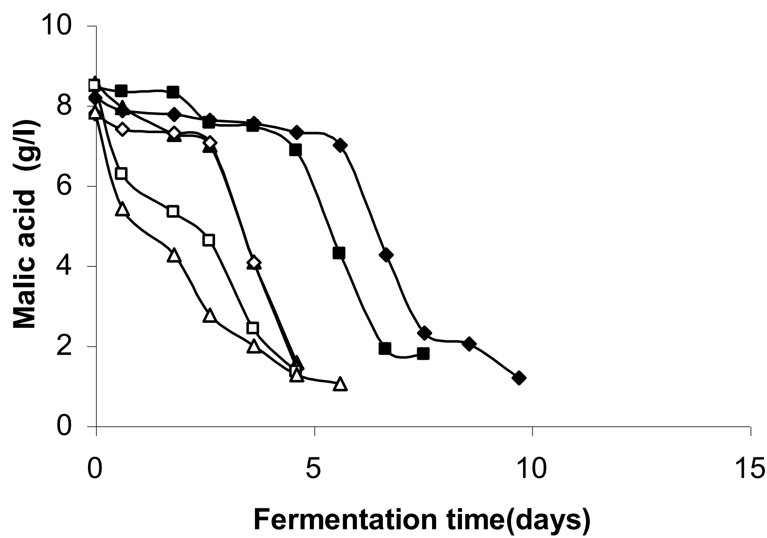


Fig.6 a,b,c : Berovic et al.







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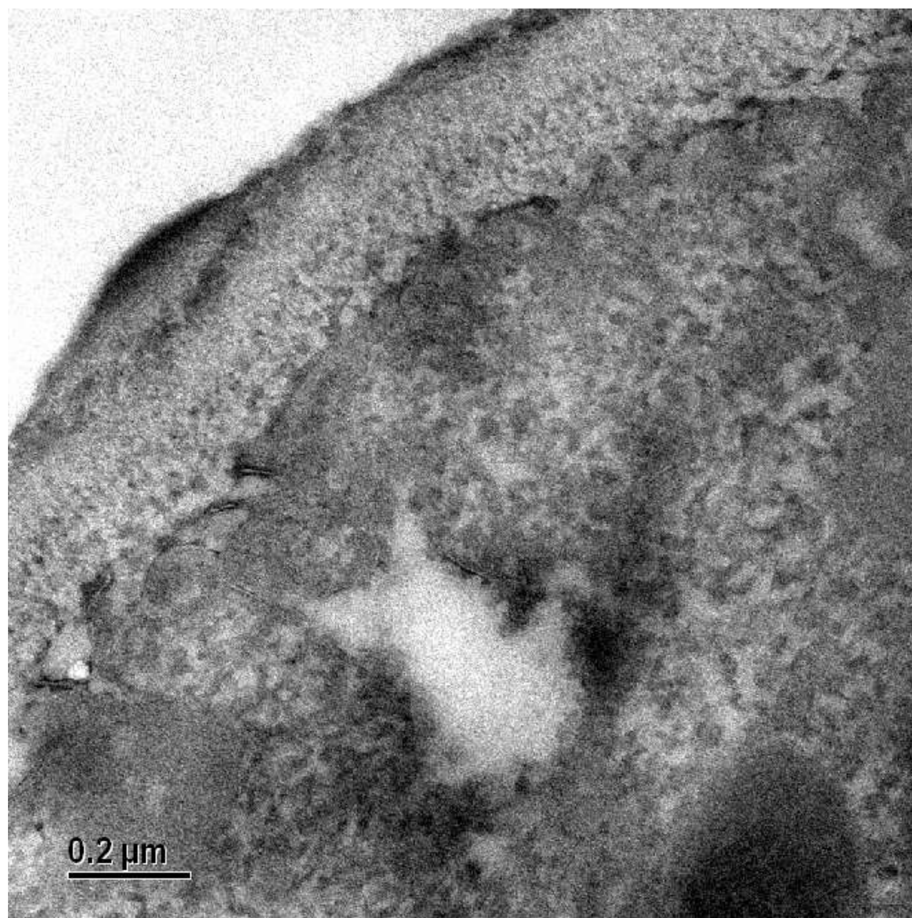

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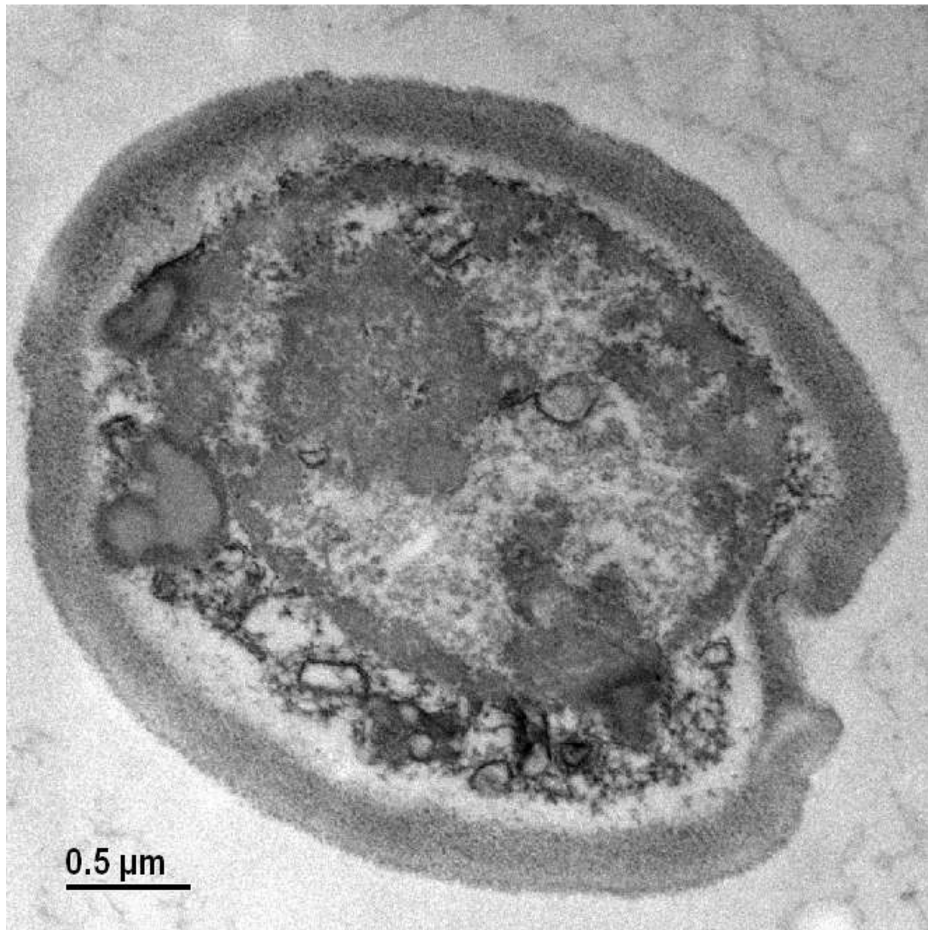

Fig.8 b : Berovic et al