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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 was studied. The results of high performance liquid chromatography (HPLC) and gas chromatography (GC) indicated 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 changes in cell morphology. The empirical experience resulting from present laboratory experiments 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 (Alternating Currant) or DC (Direct Currant) electrical current by electrostimulation with different pulse amplitudes.

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. These findings 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 a frequency of 50 Hz for *in vitro* electrostimulation of suspensions 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 $kV \cdot cm^{-1}$ was found as the critical value for the growth of *S. cerevisiae*. In electrostimulation of *Yersinia enterocolitica* Alvarez *et al.* (2003) found that increasing 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 needed for normal function of the cell. Local differences in the ionic area that resulted from 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 channels (Chen *et al.* 1998, Coster and Chilcott 1999).

Material and Methods

M i c r o o r g a n i s m : *Saccharomyces cerevisiae* yeast (Fermicru, AR2Val de Loire, France) 2 g 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 $cells \cdot ml^{-1}$ was used in all experiments.

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

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Electrostimulation: Constant (DC) electric current of different strength (1.3, 7.7, 30 µA) was applied. Pt/Ir electrodes and agar bridges were applied.

Fermentor: A 10 l stirred tank reactor of standard configuration (Bioengineering AG, Switzerland) was used. It was equipped with a 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 fermentors' head space was filled with N_2 to prevent oxidation of the fermenting grape must.

Analytical methods: 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 x 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^{-1} (0,008·10^{-3} l·s^{-1}). 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 4,000 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 0.1 M 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 measurement in the first aerobic phase at low oxygen pressure in fermenting must, shows small changes from 380-400 mV. This increase is most evident at 18 ºC, while at 26 ºC this phase is the shortest. Redox potential drop to negative values indicates that yeast is turning to anaerobic phase and to production of ethanol (Kukec *et al.* 2003). Minima in redox potential measurements were -170 mV at -120 h of fermentation (T = 18 ºC), - 185 mV at 72 h (T = 22 ºC) and - 220 mV at 48 h (T = 26 ºC). Comparing redox potential measurements of the control samples to the measurements with electrostimulation, faster course of aerobic and anaerobic phases, with more intensive microbial activities similar to those in control experiment at T = 26 ºC, were observed. All of the changes in redox potential occures already at 38 h, 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 (Fig. 1 a, b).

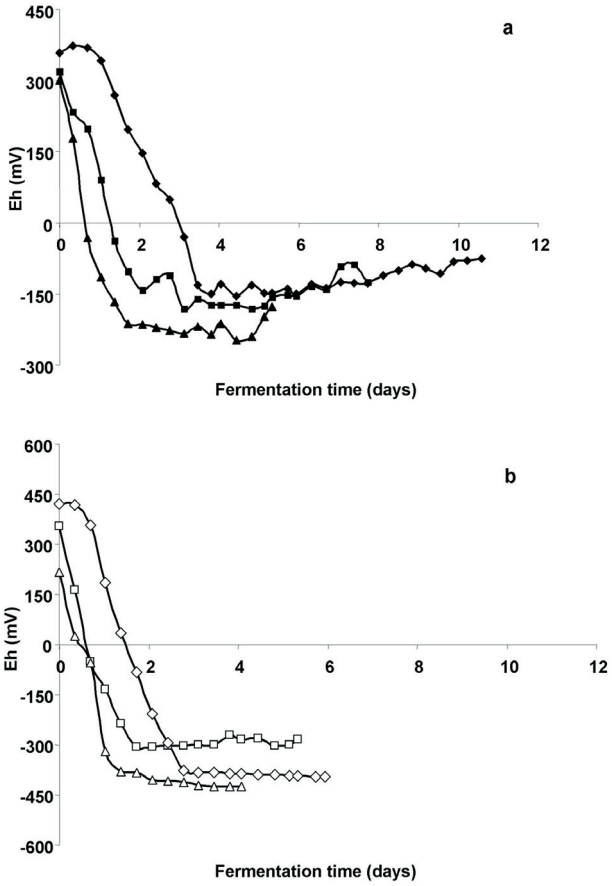


Fig. 1: Fermentation *on-line* redox potential courses. (**a**) ♦T = 18, ■22 and ▲26 °C without electrostimulation; (**b**) ◊T = 26 °C (1,3 µA), □T = 26 °C (7,7 µA), ΔT = 26 °C (30 µA) with electrostimulation.

Biomass production was highest in the samples with electrostimulation. After 10 d at the end of fermentation it was 4.5 g·l^{-1} at 1.3 µA, 5.5 g·l^{-1} at 7.7 µA and 6,2 g·l^{-1} 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 a 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 more slow and a rest of fructose of 16.6 g·l^{-1} and 11.6 g·l^{-1} was detected (Fig. 3 a, b).

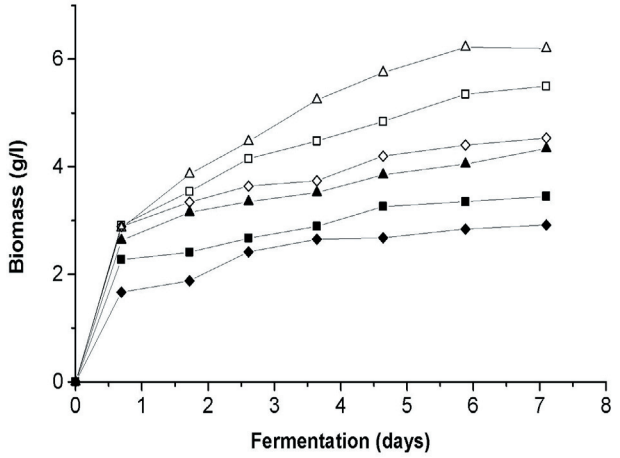
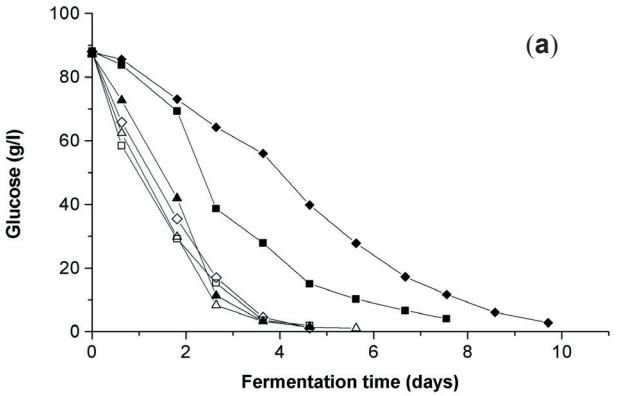


Fig. 2: Biomass production. ♦T = 18 °C , ■T = 22 °C, ▲T = 26 °C, without electrostimulation; ◊T = 26 °C (1,3 μA), □T = 26 °C (7,7 μA), ΔT = 26 °C (30 μA) with electrostimulation.



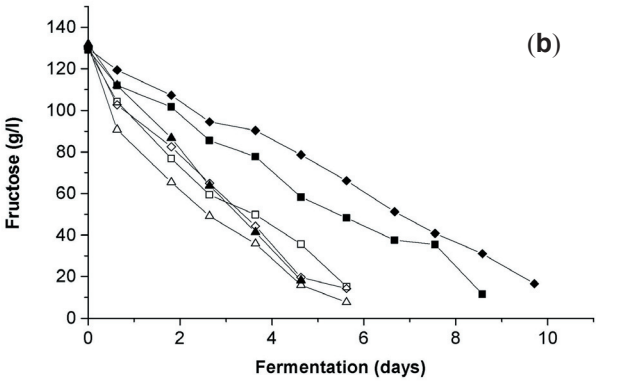


Fig. 3: (**a**) glucose consumption, (**b**) fructose consumption. ♦T = 18 °C, ■T = 22 °C, ▲T = 26 °C without electrostimulation ◊T = 26 °C (1,3 μA), □T = 26 °C (7,7 μA), ΔT = 26 °C (30 μA) with electrostimulation.

Acetaldehyde production in non stimulated experiments at 18 and 22 ºC reached on average 16 mg·l^{-1} in 4 d and 20 mg·l^{-1} at 26 ºC in 60 h. In the same period in electrostimulated samples acetaldehyde production highly increased to 28 mg·l^{-1} (1.3 μA), 31 mg·l^{-1} (7.7 μA) and and 33 mg·l^{-1} (30 μA). In all these experiments, conversion of acetaldehyde to ethanol was also much faster. It was mostly converted to ethanol in 5 d, while at lower temperature (18 and 22 ºC) control samples this process was more slow and lasted up to 10 d (Fig. 4).

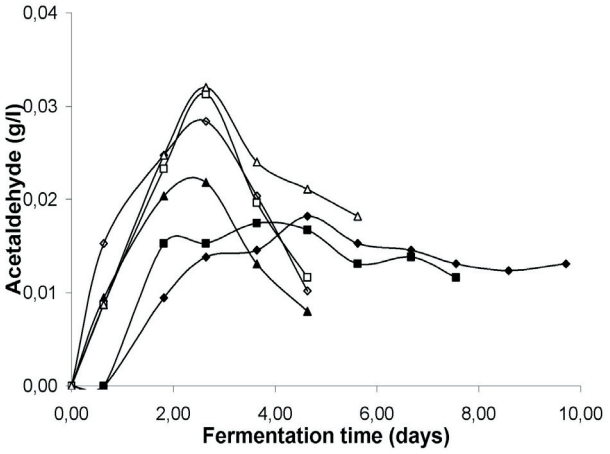


Fig. 4: Acetaldehyde in fermentation time course. ♦T = 18 °C, ■T = 22 °C, ▲T = 26 °C without electrostimulation, ◊T = 26 °C (1,3 μA), □T = 26 °C (7,7 μA), ΔT = 26 °C (30 μA) with electrostimulation.

Ethanol production, in all of the control experiments was as follows: after 10 d of fermentation at 18 ºC 60 g·l^{-1} were produced, 78 g·l^{-1} at 22 ºC after 8 d and 80 g·l^{-1} in the control at 26 ºC after 5 d. With electrostimulation at low current of 1.3 μA ethanol production after 4 d was 85 g·l^{-1}, while at higher currents of 7.7 μA it was 95 g·l^{-1} and 99 g·l^{-1} at 30 μA (Fig. 5 a). 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^{-1} of glycerol production after 6 d. At a higher current of 30 μA in the same time 10 g·l^{-1} of glycerol was detected. (Fig. 5 b).

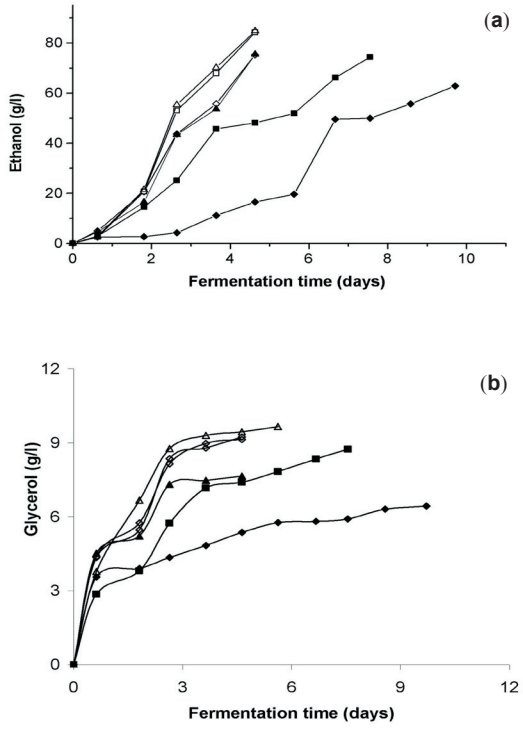


Fig. 5: (**a**) production of ethanol, (**b**) production of glycerol, ♦T = 18 °C, ■T = 22 °C, ▲T = 26 °C, without electrostimulation ◊T = 26 °C (1,3 μA), □T = 26 °C (7,7 μA), ΔT = 26 °C (30 μA), with electrostimulation.

Metabolic activity of wine yeast was also controlled by measuring isoamyl alcohol, 1-propanol and 2- butanol accumulation. While at 26 ºC in the control as well as in a low current field of 1.3 µA, 75 g·l^{-1} of isoamyl alcohol was obtained, at higher currents of 7.7 and 30 µA 85 g·l^{-1} were produced in 5 d. At higher currents of 7.7 and 30 µA also higher amounts of 1-propanol (45 g·l^{-1}) and of 2- butanol (49 mg·l^{-1}) were obtained in 5.5 d (Fig. 6 a, b, c).

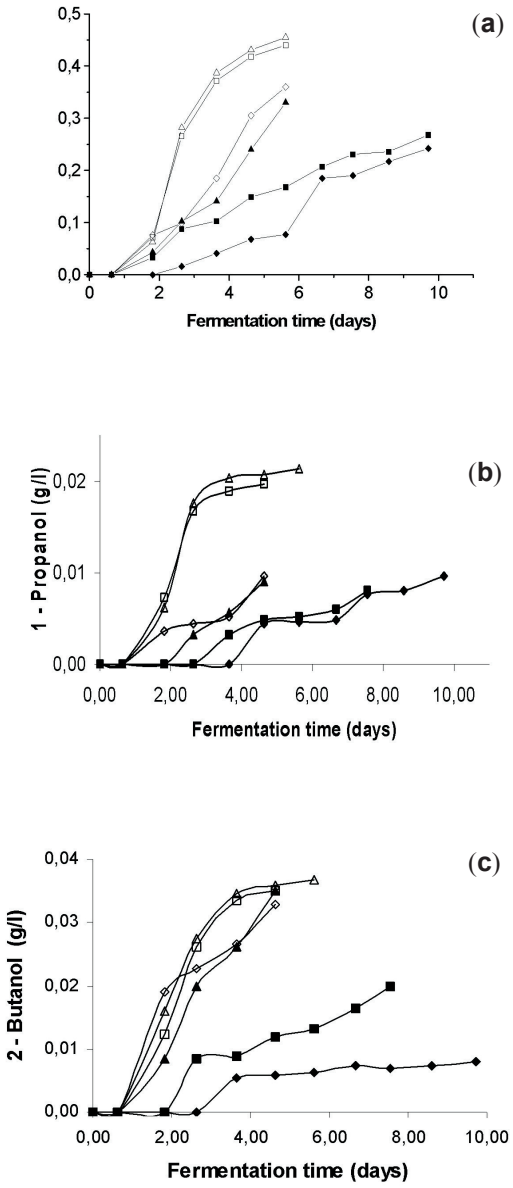


Fig. 6: (**a**) accumulation of iso-amyl alcohol, (**b**) accumulation of 1-propanol, (**c**) accumulation of 2-butanol, ♦T = 18 °C, ■T = 22 °C, ▲T = 26 °C, without electrostimulation, ◊T = 26 °C (1,3 µA), □T = 26 °C (7,7 µA), ΔT = 26 °C (30 µA) with electrostimulation.

In measurements of organic acids the amount of tartaric acid (5.8 g·l^{-1} at final) was nearly unchanged in all the cases of electrostimulation while its conversion rate in comparison with the control was much faster. Also malolactic conversion to lactic acid proceeded up to 4.4 g·l^{-1} in 5.5 d in all experiments where a galvanic field was applied and 50 % higher yield was produced (Fig. 7 a, b, c).

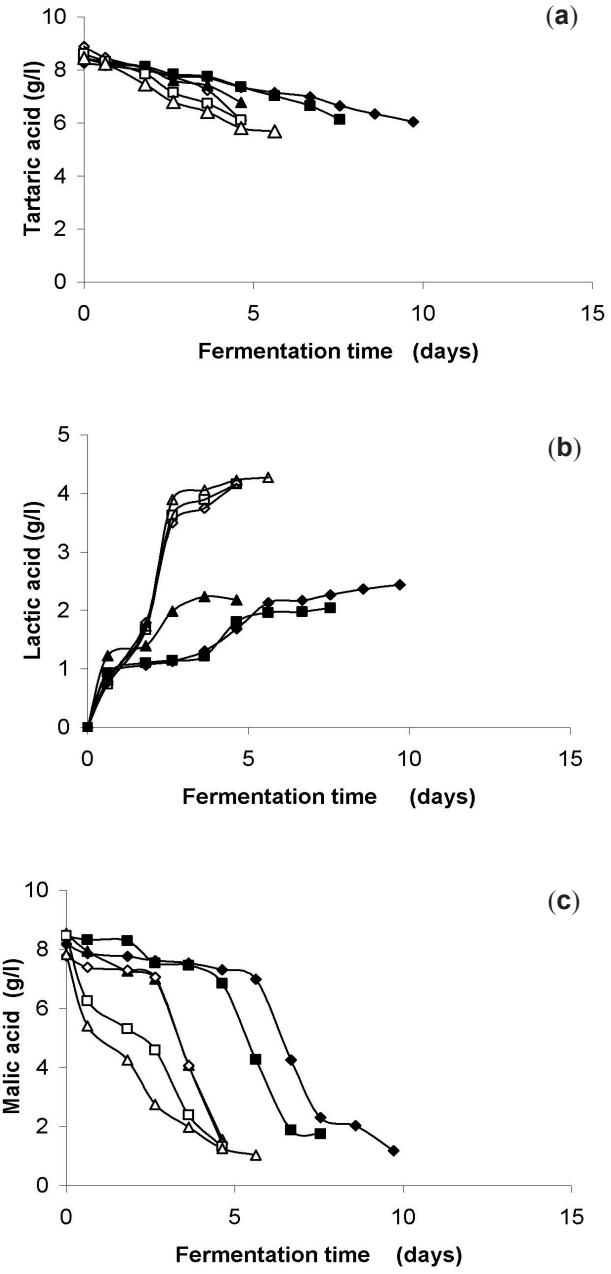


Fig. 7: (**a**) Tartaric acid time course, (**b**) malic acid time course, (**c**) accumulation of lactic acid ♦T = 18 °C, ■T = 22 °C, ▲T = 26 °C, without electrostimulation ◊T = 26 °C (1,3 µA), □T = 26 °C (7,7 µA), ΔT = 26 °C (30 µA) with electrostimulation.

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

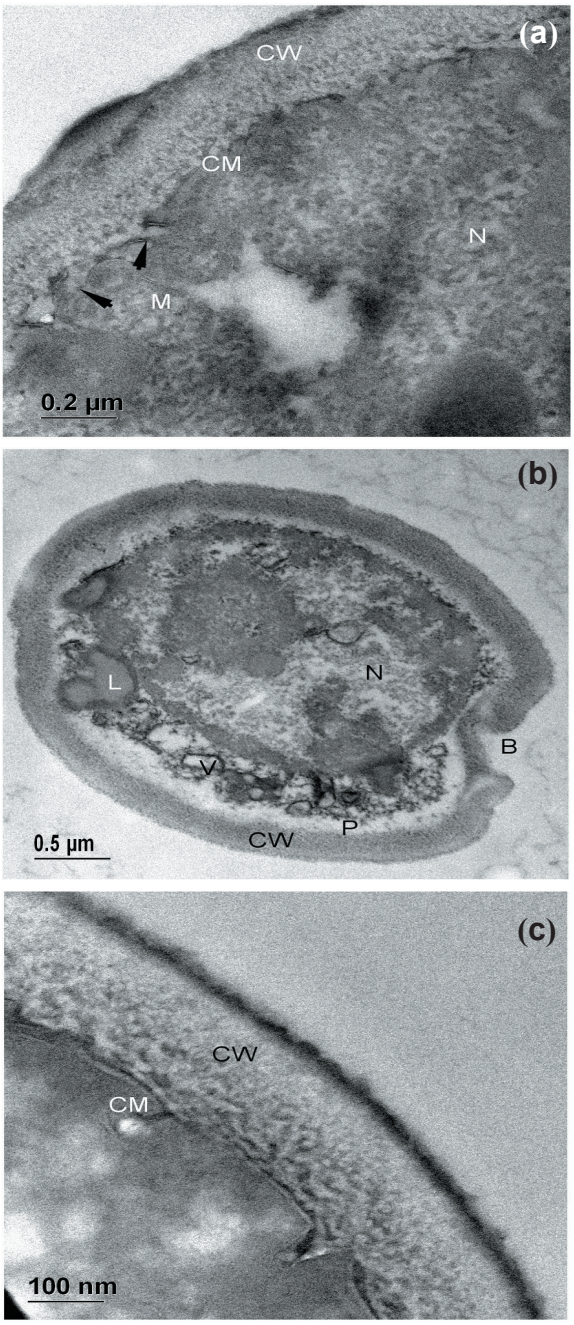


Fig. 8: TEM micrographs of a yeast cell (**a**) with a cell wall (CW) composed of fibrillar layer and periplasm (P). The nucleus is surrounded by lipid globules (L), vacuoles (V) and mitochondria (M). The invaginated area is the site of bud formation (B). Details of cell surface (**b**, **c**) with cell wall and cell membrane (CM) forming invaginations (arrows).

and microbial cell regeneration. Various biological liquids consist of a large amount of water, ions, polar molecules, charged proteins, lipids, hormons and colloid 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 (Barnes 1985, Omori *et al.* 1997).

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, the nucleus was surrounded by vacuoles and lipid droplets. The cell membrane was deeply invaginated at certain areas which could be related to increased kinetics in electrostimulated cells.

Direct electric current (DC) of 7.7 and 30 µA was found as a promoting factor stimulating faster and more intensive cell metabolism. Application of electro-stimulation in grape must showed an increase in the rate of alcohol fermentation at 18 ºC and, as in most of the cases, 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 a temperature of 25 ºC. Apart from 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. The most remarkable increase can be seen with lactic acid and glycerol, one of the most relevant compounds that contributes to smoother wines and more complexity of the wine. Presented results are a new approach to grape musts alcohol fermentation that could significantly contribute to the quality of wine and its process control.

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