The World´s first continuous in-line measurement system for detection of central parameter of bacterial cells

The EloTrace by Biotronix

EloTrace is a fully automated measurement unit. From any fermenter, be it stainless, glass, disposible plastic or a smaller cultivation vessel, samples are being continuously taken, prepared and analyzed by use of a sterilizable tube bypass. Profile graphs and numbers for AP levels, cell size are being created in real time and at the same time correlated with saved data from prior fermentation runs. The generated report files document all measured aspects of the development of the fermentation. The system does not use any reagents, chemicals or other consumables and is therefore very affordable in terms of running costs.

With EloTrace, the whole measurement is fully automatic and in-line. For each individual measurement, the EloTrace is being fed a sample automatically from an autoclavable tube bypass; the sample will be also fully automatically prepared by predilution to 2 ml of suspension with a concentration of $5*10^8$ cells/ml (OD=0.1). Based on this automatic pre-dilution step EloTrace can process and precisely measure even suspension samples with extremely high OD/Biomass values on a continuous basis.

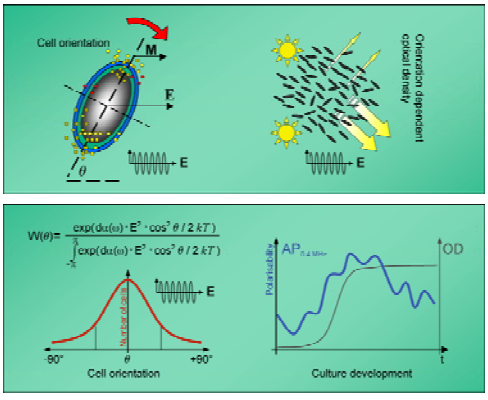
Electrooptical Analysis of Bacteria in suspension culture

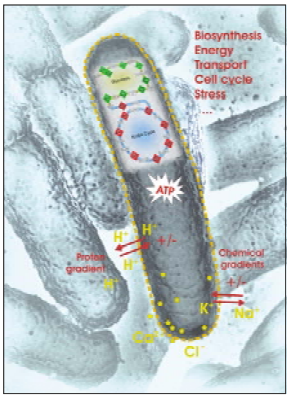
The scientific working principle, the nature of the signal

The measurement principle

The patented electrooptical measurement technology employed in our novel EloTrace analysis instrument is a combination of two working principles:

The creation of a very sensitive electrical current field is coupled with the subsequent **photometric analysis** of the effect of the electrical field on the individual bacteria cells. The weak electrical AC voltage fields are having an **polarizing effect** on the **ions** in the cell cytoplasm, which are unable to leave the cell through the membrane due to the membrane´s electro-physiological properties. Therefore the charged ions concentrate near the membrane in their try to orient themselves in the electrical field.





This in turn leads to a minuscule force effecting each cell to slightly change its spatial orientation within the suspension flow. This change in orientation correlates directly with the free ion concentration in the cytoplasm and is being picked up extremely sensitively and precisely by our proprietary high-end photometric sensors.

The result parameters

Each measurement with the EloTrace instrument simultaneously delivers main independent parameters concerning the analyzed bacteria, which accurately reflect the current physiological and morphological state of the sample cells:

- The degree of „Polarizability“ of electrical charges in both cytoplasm and membrane, which we named „**AP“ – value** (physically: *Anisotropic Polarizability*), which directly reflects the ions distribution on cell structures and intracellullar ionic pool (lead to information about: transport activity, energy level of membrane, intracellular metabolic flux, inhibition/stress level)
- Cell Size/Length of bacteria with the typical elongated cell morphology
- Biomass concentration (optical density measurement)
- Cell concentration (cell/ml)

AP-level

The physical measurement principle is based on the polarizability of electrical charges/ions at the intact cell membrane („Maxwell-Wagner Effect“). The „**AP**“ level essentially reflects the degree of the change of the geometrical orientation of the bacteria cell within the suspension as a reaction to the created electrical current field during measurement.. The **AP** level directly correlates with the concentration of ions (free ions and charged metabolic intermediates) within the cytoplasm of the cell. This pool of mobile ions participates in intracellular transport activity as well as in the energy status of the cell membrane.

With bacteria cells therefore these **AP** values reflect the current activity/amount of "metabolic pathways", "pathway capacity" and, in general, the energy status of the cell. All measurement parameters are based on the values of the statistically average, prototypical cell in the sample and therefore represent a direct, cell based measurement.

Intracellular biochemical reactions are being accelerated by the decrease of inhibitions or limitations, by increased substrate uptake, active transport activity as well as the production of proteins or enzymes (induced by stress or deliberate induction). All these cellular processes have a positive influence on the energy level of the membrane and the concentration of the ions participating in these activities, which is directly reflected by a high **AP**-level. The same is true for inactive cells with a high energy level, be it based on an intact transport system, the absence of toxic influences or stable proton gradients.

In essence, it can be said that a cell (culture) with a high **AP**-level has an excellent potential for stable growth and high productivity.

Likewise, negative developments like adverse pH-levels, a decrease in O_2 or substrate uptake, damage or „depolarization“ of the cell membrane are directly reflected in a decrease of the **AP**-levels.

Cell size, Cell length

The analysis method employed in the EloTrace Analyzer has been specifically optimized for bacteria cells with a length between 0,8 - 12 µm and their typical elongated cell morphology. Thanks to the full automation of the measurement as well as the high sensitivity of the employed measurement technology (exploiting the knowledge how elongated particles „relax“ in liquid environments) lead to an unprecedented precision and reproducibility of the measurement results compared to conventional measurement principles.

This accurate knowledge about changes in cell size in the culture are of paramount importance, since changes / adaptations in cell volume in reaction to their environmental conditions and intracellular processes have a direct effect on the regulatory mechanisms of bacteria cells. Cell size delivers valuable information about cellular osmolarity, cell division, stress based elongation or exhaustion of the cells and therefore constitutes a vital quality parameter for a bacteria culture.

Biomass concentration (optical density measurement)
According to automatic sample dilution works EloTrace with arbitrary OD-value. Also by high cell density fermentation an automatic on-line biomass control is secured.

Cell concentration
The determination of the cell concentrations (cell/ml) is based on the determined OD-value and the measured cell size.

Inhibition / Metabolic Regulation

The growth of *E. coli* is often accompanied by a so called "overflow"-metabolism. For instance, the „by-product" Acetic Acid causes a vehemently negative influence on the physiological status of the cells. The shifts in the intracellular ion balance, the resulting metabolic regulations and the effects on cytoplasmic osmolarity have been described e.g. by Roe A. J. et al. 1998* and Wolfe, A. J. 2005**.

With the use of EloTrace the metabolic regulations caused by the Acetic Acid (time, phases, intensity of inhibition, adaptation) can be detected and monitored. The changes in metabolic flux as well as their effect on cell size can be identified on-line during the growth of an *E. coli* culture. Based on that knowledge, the inhibiting effects of toxic metabolites can me minimized by controlling the process accordingly. One could say that EloTrace can be used as a „Viewer" of the actul current physiological cell status.

One of the main inhibitors for the growth of Lactobacilli is the created Lactic Acid. As an example, the changes of the physiological and morphological properties of the cells caused by Lacic Acid can be shown for *L. plantarum.* By virtue of the electrooptical monitoring it is possible to identify those process phases in which the cells have a higher tolerance against lactic acid at a moderate stress level due to respective adaptation.

This way, the highest yield of starter cultures with high vitality and storage stability can be ensured.

*- Roe A. J. et al. (1998) Perturbation of anion balance during inhibition of growth of *Escherichia coli* by weak acids. J. Bacteriol. 180:767-772.
**- Wolfe, A. J. 2005. The acetate switch. Microbiol. Mol. Biol. Rev. 69:12-50.

Fields of application

- Production of recombinant proteins or other drugs (recombinant human insuline, Interferone etc.)
- Production of bacterial vaccines: bacteria with reproducible vitality or consistant proteome patterns
- Production of starter cultures, bacterial protection cultures, probiotic cultures
- Production of biological substances:Herstellung von Biosubstanzen: technical enzymes, fine chemicals, aminoacids, new materials, „white" biotechnology
- Prozess control: Monitoring of biosynthesis activity, Optimization of fermentation conditions, Controlling
- Quality control of the manufacturing of bacterial vaccines, GMP-Controlling
- Biomedical, biotechnological R&D

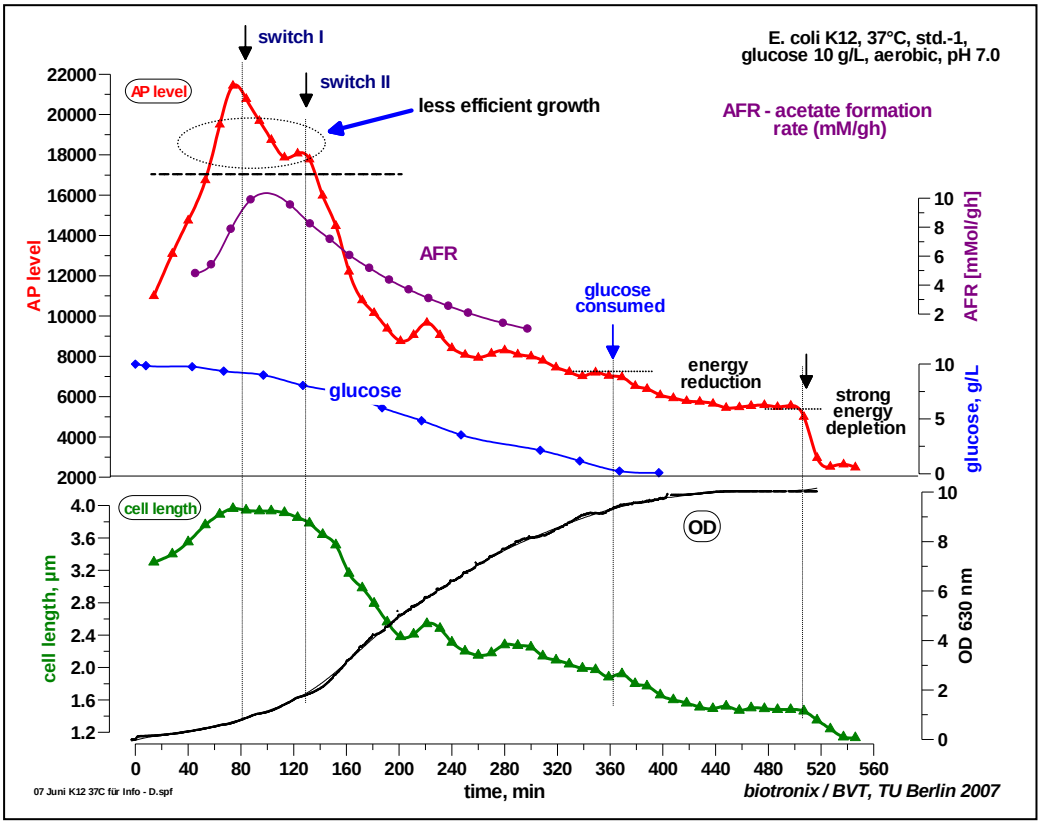
Product Benefits for process control

- Achievement of balanced growth, Avoidance of „overflow"-metabolis
- Improved Understanding about growth behaviour and critical phases of a fermentation process
- Determination of the ideal time for harvesting with a maximized yield of stable/active cells
- Reproducible yields
- High degree of automation
- Measurements *in vivo* and on-line
- Quality Control through novel go/no go-criteria
- Comprehensive continuous documentation
- Increase of product quantity and quality of fermentation runs

Example I: EloTrace used for characterization of growth phases of *E. coli* K12

Task at hand: Identification of critical time points during fermentation, Identifying optimized end of process (max. yield of stable and active cells), comprehensive process documentation.

- Test fermentation at the Institute for Bioprocess Technology of Berlin Technical University. Growth of *E. coli* K12 in complete medium with glucose, pH 7.0, aerobic (pO_2 70%).



State of **starter culture**: high AP-Levels, "normal" cell size – very active, uninhibited cells, high growth potential with a short phase of „lag".

After ca. 60 min: steep increase of AP, highest respiratory activity and substrate uptake per cell, highest biomass increase per time unit (however: not per gram of substrate), Side products of substrate decomposition, primarily Acetic acid (ES), are increasingly intracellularly generated and excreted. However, the excretion of those Acetic acid anions does not pose a problem yet, since the extracellular ES-concentrations are still low (otherwise reduction of AP level).

After 80 min (switch I): AP-values decrease, first correction of metabolic flux, growth rate of *E. coli* is slightly reduced.

Phase 80-120 min: Highest ES-creation rate of 10 mM/g biomass/h). After 120 min the extracellular concentration of ES reaches a critical level of ca. 2,5 mM. ES-excretion becomes more difficult.

130 min (switch II): Further regulation of Substrate flux (Reduction of uptake of substrate and O_2, reduction of biomass formation) caused by the inhibiting effect of Acetic Acid (known effects: Shifting of the intracellular ion balance, of –pH, of the membrane potential and the osmolarity). The electro-optical measurements show that the "switch"-effects described herein are depending on the concentration of extracellular metabolites. The respective AP-profiles are reproducible and very specific for the individual process.

After 130 min: increasingly inhibiting effect caused by increasing acetic acid concentration, lowering of substrate uptake, the growth rate decreases. There are regular „waves" of cell activity based on the fine regulations of the metabolism. The time, duration and intensity of these reactions can be determined precisely and reproducibly by the EloTrace, which is very useful for determining the ideal time for sampling for additional off-line analyses, eg. Proteome Analytics.

320-340 min: ideal phase for harvesting with an optimized yield of stable and active cells

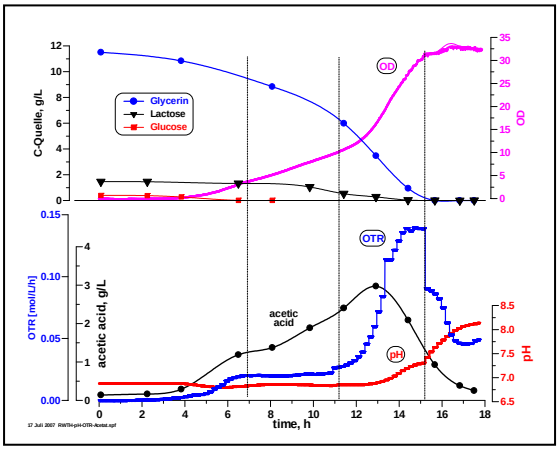
After 370 min: Glucose is totally consumed, Acetic Acid reaches a maximum concentration of 21,8 mM and is taken up again by the cells. Characteristic decrease of the AP-values caused by a decrease of the energy level of the cells. Accelerated decrease in cell activity! If used as an inocolum these cells would show a significantly longer „lag“ phase, slower growth and in general a defective process development.

After 500 min: the exhausted cells are not able to generate enough energy to keep up the required membrane potentials. Viability drops steeply.

Example II: EloTrace for Monitoring of processes with Induction (Protein expression), Autoinduction System Overnight Express™, Novagen

Task at hand: Achievement of optimized cell growth with at the same time minimal „overflow“ to avoid the effect of "low yield, low quality", strong protein expression, stable synthesis performance, determination of the ideal harvesting time. Creation of a comprehensive informative process documentation.

Fermentation at the Institute for Bioprocess Technology, RWTH Aachen University (Prof. Büchs). Recombinant E. coli Strain, Autoinduction System Overnight Express™ medium by Novagen.



Phase A: Adaptation of the cells to the fresh medium, Building up of the Membrane Potential and the active Transport system. „Instable“ Starter Culture.

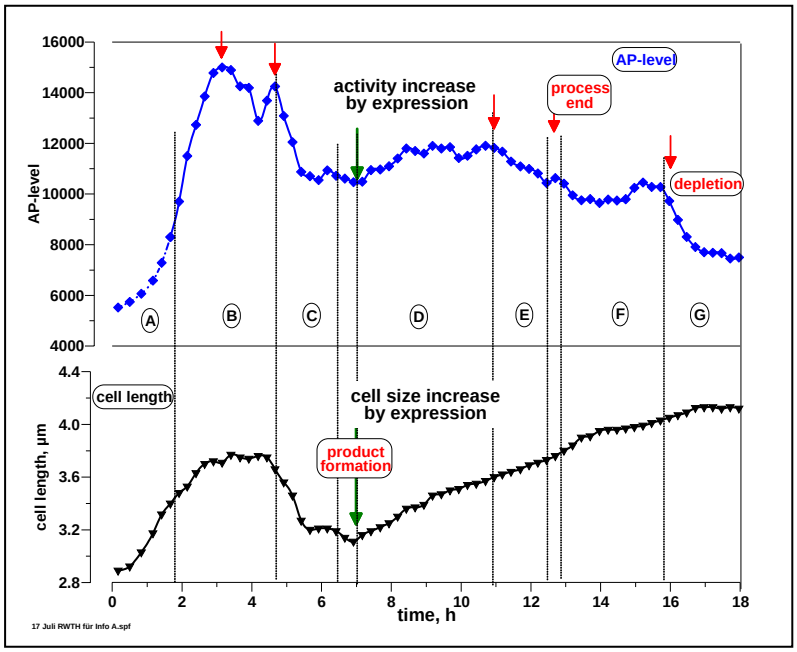
Phase B: highest energy level of the membrane, highest substrate uptake per cell and fastest growth phase not necessarily directly linked to Substrate concentration, highest cell volume. By-products of the metabolic activity consist primarily of Acidic Acid (ES).

After 3 hours: small correction of metabolic flux.

Phase C (after 4,5 h): Characteristic reduction of the AP-values as well as of cell size. The strong effect of the „overflow metabolism“ leads to a decrease of substrate uptake and growth (see description in **example I** given above).

6,5 h: Induction by Lactose.

After 7h: a progressive increase in AP-values shows an increase in metabolic activity and synthesis performance. Without the induction it would have been to be expected that AP-values decrease. Analytical methods also showed after 7 h the first creation of product.



The characteristical size growth of the host cells is caused by a disturbance of cell division which in turn is caused by the expression, the formation and intracellular accumulation of the target protein. These effects could be precisely determined and documented with the EloTrace in other fermentations of recombinant *E. coli* as well (Insulin, α-Interferon).

After 11 h: Decrease of AP-values based on a decrease in the effect of the induction and of the synthesis performance.

About after 12,5 h: ideal time for harvesting.

After ca. 13 h: The fermentation run experiences an overgrowth of the culture by non-productive, plasmid free cells.

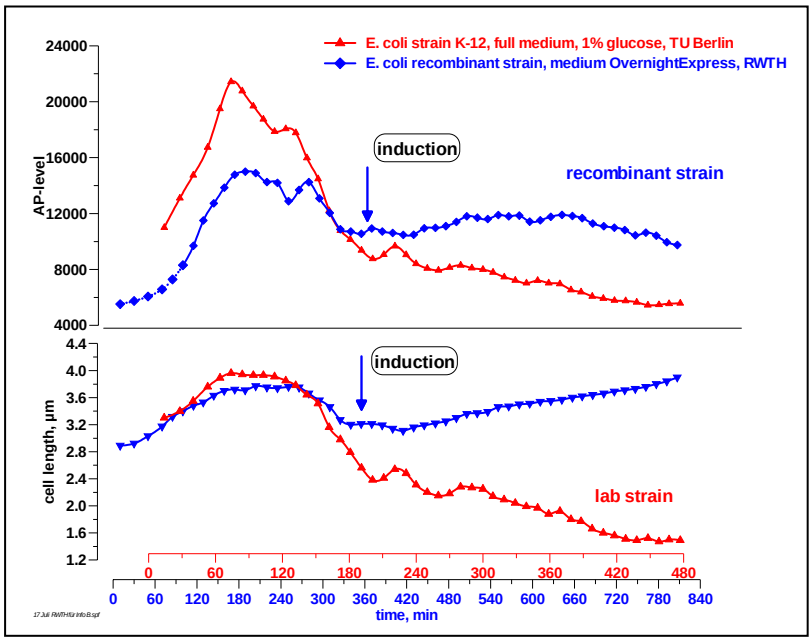
14-16 h: A very marked AP-peak, induction of an Enzyme system for the Acetate metabolism.

After 16 h: steep decline of AP-Level caused by progressive exhaustion of *E. coli*. Use of the Acetate as last remaining energy (C) source coupled with a decrease of the energy status of the cells.

Example III: Profile with / without Protein expression

Comparison between growth profiles with protein expression (see above **example II**) versus an unmodified *E. coli* K12 strain (see above **example I**).

The characteristically change of physiology as well as morphology after the occurrence of the induction can be easily recognized. For better comparison, the time scale of the lab strain K12 (red) was synchronized with the time scale for the recombinant strain (blue).



Conclusion regarding the benefits of EloTrace in process control:

- **The current physiological state of the bacteria is continuously measured on-line, which is ideal for process documentation and scale-up.**
- **Knowledge about and understanding of growth behaviour as well as critical phases in the fermentation development are greatly increased**
- **Detection of the occurrence of „overflow metabolism“ is made possible. The inhibiting effect of toxic metabolites is directly taken from the analysis of the actual cells as opposed to from the media suspension**
- **The perfect time for harvesting leading to an optimized yield of stable and active cells can be determined with unmatched precision.**

InvestitionsBank des Landes Brandenburg

Dieses Projekt wird aus Mitteln des Ministeriums für Wirtschaft des Landes Brandenburg und der EU gefördert