Electrooptical measurements as process analytical tool for biogas processes: comparative study with Flow Cytometry

Master thesis

Danai-Kalliopi Moschidi
This work was done at the Institute for Biotechnology,
Department of Bioprocess Engineering,
Technical University of Berlin, Germany

Supervisors:
Dr.-Ing. Stefan Junne and Prof. Gerasimos Lyberatos
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Abstract

Due to rising costs, constant dwindling fossil energy sources, and environmental concerns regarding extraction and use of petroleum and natural gas over the last decades it is considered necessary to shift to the renewable energy sources. Bioenergy and renewable raw materials are two of some economically competitive alternative sustainable energy sources in Germany and in Greece. In Europe, Germany is the biggest biogas production and has the most advanced biogas technology, whereas the biogas production in Greece has not been developed to the extent. A suitable renewable energy source is the biogas which typically refers to a mixture of different gases produced by the breakdown of organic matter in the absence of oxygen. The appropriate conversion and process management technologies need to be optimized to ensure a predefined quality at the end of the manufacturing process. The appropriate measurement program and technologies which design, analyze and control manufacturing processes was defined the Process Analytical Technologies (PAT). In this study, Flow Cytometry and Electrooptical measurements are used as process analytical tools.

Flow Cytometry is a laser-based technique used in biotechnology to assess biological and physiological characteristics of several types of cells. Basically, a flow cytometer composed of the fluidics system, the optics system and the electronics system. Different dyes are used, which allows the analysis of specific cellular characteristics at individual cells. There are a variety of fluorescent dyes with different properties which have to be selected according to the purpose of the investigation. In this study, DAPI, BOX, SYTO 13 and PI are applied for staining biogas cells. EloTrace is an automatic electrooptical measurement system for detection of parameters of bacterial cells and the most important application is the monitoring of biosynthesis activity, the optimization of fermentation conditions and the process control of fermentation.

In this study, the optimization of staining conditions for DAPI was done using a wild-type strain of genus as a model bacterium, *Clostridium acetobutylicum* ATCC 824. The determination of the variables and their sensitivity for this optimization was performed by Design of Experiments (DoE) and the outcome of two experimental designs was that the optimal conditions for DAPI staining are 37°C as staining temperature, 20 µg/mL as final concentration of DAPI and 30 minutes as staining time. The staining conditions of the three dyes PI, BOX and SYTO 13 for biogas samples were based on protocol for Corynebacterium glutamicum cultivations.

The application of Flow Cytometry and Electrooptical Measurements for the analysis of biogas samples requires previous sample purification. In this study, five different procedures to prepare the biogas samples were investigated. Regarding to the Flow...
Cytometry and Electrooptical results, the conclusion for the preparation of biogas samples was that an easier and lower costly treatment had similar results with a more complicated procedure.
Περίληψη

Λόγω της αύξησης του κόστους, της συνεχούς εξάντλησης των ορυκτών πηγών ενέργειας, καθώς και των περιβαλλοντικών ανησυχιών σχετικά με την εξόρυξη και τη χρήση του πετρελαίου και του φυσικού αερίου, θεωρείται αναγκαίο η στροφή στις ανανεώσιμες πηγές ενέργειας. Η βιοενέργεια και οι ανανεώσιμες πηγές ισχύος ύλες είναι δύο οικονομικά ανταγωνιστικές εναλλακτικές βιώσιμες πηγές ενέργειας στη Γερμανία και στην Ελλάδα. Στην Ευρώπη, η Γερμανία είναι η χώρα με την μεγαλύτερη παραγωγή βιοαερίου και έχει την πιο προηγμένη τεχνολογία, σε αντίθεση με την παραγωγή βιοαερίου στην Ελλάδα, η οποία δεν έχει αναπτυχθεί σε αυτόν τον βαθμό. Μια κατάλληλη ανανεώσιμη πηγή ενέργειας είναι το βιοαέριο, το οποίο αναφέρεται σε ένα μείγμα διαφορετικών αερίων που παράγονται από τη διάσπαση της οργανικής ύλης χαρακτηριστικών του οξυγόνου. Οι κατάλληλες τεχνολογίες μετατροπής και διαχείρισης της διαδικασίας πρέπει να βελτιστοποιηθούν για να εξασφαλιστεί μια προκαθορισμένη ποιότητα στο τέλος της διαδικασίας παραγωγής βιοαερίου. Ως το κατάλληλο πρόγραμμα μέτρησης και των τεχνολογιών του για την διερεύνηση της ανάλυση και τον έλεγχο των διαδικασιών παραγωγής ορίστηκαν οι Τεχνολογίες Αναλυσης Διεργασιών, Process Analytical Technologies (PAT). Σε αυτή τη μελέτη, η κυτταρομετρία ροής και οι ηλεκτροπληρές μετρήσεις χρησιμοποιήθηκαν ως Εργαλεία Αναλυσης Διεργασιών.

Η κυτταρομετρία ροής είναι μία τεχνική που χρησιμοποιούνται στη βιοτεχνολογία για την αξιολόγηση των βιολογικών και φυσιολογικών χαρακτηριστικών των διαφόρων τύπων των κυττάρων. Ένα τυπικό κυτταρόμετρο ροής αποτελείται από το σύστημα των ρευστών, το οπτικό σύστημα και το ηλεκτρονικό σύστημα. Διαφορετικές χρωστικές χρησιμοποιούνται, οι οποίες επιτρέπουν την ανάλυση των ειδικών κυτταρικών χαρακτηριστικών σε μεμονωμένα κύτταρα. Υπάρχει μια ποικιλία από φθορίζουσες χρωστικές με διαφορετικές ιδιότητες, οι οποίες θα πρέπει να επιλέγονται σύμφωνα με τον σκοπό της έρευνας. Στην παρούσα μελέτη, τα DAPI, BOX, SYTO 13 και PI εφαρμόζονται για τη χρώση των κυττάρων βιοαερίου. Το EloTrace είναι ένα αυτόματο σύστημα μέτρησης ηλεκτροπληρών για την αναλυτική των παραμέτρων των βακτηριακών κυττάρων και οι σημαντικότερες εφαρμογές του είναι η παρακολούθηση της δραστηριότητας της βιοσύνθεσης, η βελτιστοποίηση των συνθηκών ύλεως και ο έλεγχος της διαδικασίας της ύλεως.

Σε αυτή τη μελέτη, η βελτιστοποίηση των συνθηκών ύλεως για την χρωστική DAPI έγινε χρησιμοποιώντας ένα στέλεχος του γένους Clostridia ως πρότυπο βακτήριο, το Clostridium acetobutylicum ATCC 824. Ο προσδιορισμός των μεταβλητών και την ευαισθησία τους για την παρούσα βελτιστοποίηση διεξήχθη με τον Σχεδιασμό Πειραμάτων, Design of Experiments (DoE) και το αποτέλεσμα των δύο πειραματικών
σχεδιασμών που έγιναν ήταν ότι οι ιδανικές συνθήκες για την χρωστική DAPI είναι 37°C ως θερμοκρασία χρώσης, 20 μg/ml ως τελική συγκέντρωση του DAPI και 30 λεπτά ως χρόνος χρώσης. Οι συνθήκες χρώσης των άλλων τριών χρωστικών, PI, BOX και SYTO 13 για τα δείγματα βιοαερίου βασίστηκαν στο πρωτόκολλο για καλλιέργειες του βακτηρίου Corynebacterium glutamicum. Η εφαρμογή της κυτταρομετρίας ροής και ηλεκτροπτικών μετρήσεων για την ανάλυση των δειγμάτων βιοαερίου απαιτεί προηγούμενο καθαρισμό του δείγματος. Στη μελέτη αυτή, πέντε διαφορετικές διαδικασίες για την παρασκευή των δειγμάτων βιοαερίου ερευνήθηκαν. Με βάση τα αποτελέσματα της κυτταρομετρίας ροής και των ηλεκτροπτικών μετρήσεων, το συμπέρασμα για την προετοιμασία των δειγμάτων βιοαερίου ήταν ότι μια ευκολότερη και λιγότερο δαπανηρή διαδικασία είχε παρόμοια αποτελέσματα με την πιο περίπλοκη διαδικασία που χρησιμοποιήθηκε.
## List of Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>AC</td>
<td>electrical Alternating Current voltage</td>
</tr>
<tr>
<td>AP</td>
<td>Anisotropic Polarizability</td>
</tr>
<tr>
<td>BOX</td>
<td>bis-(1,3-dibutylarbituric acid)-trimethine oxonol such as; diBAC4 (3) (Deere, Porter et al. 1995), BOX (Want, Thomas et al. 2009), BOX, bis-oxonol, BOX, and DiBAC₄</td>
</tr>
<tr>
<td>BP</td>
<td>BandPass filters</td>
</tr>
<tr>
<td>C. acetobutylicum</td>
<td><em>Clostridium acetobutylicum</em></td>
</tr>
<tr>
<td>CGM</td>
<td>Clostridium Growth Medium</td>
</tr>
<tr>
<td>CHP</td>
<td>Combined Heat and Power</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical oxygen demand</td>
</tr>
<tr>
<td>CPP</td>
<td>Critical Process Parameters</td>
</tr>
<tr>
<td>CQA</td>
<td>Critical Quality Attributes</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>DoE</td>
<td>Design of Experiments</td>
</tr>
<tr>
<td>EBA</td>
<td>European Biogas Association</td>
</tr>
<tr>
<td>EEG</td>
<td>Renewable Energy Sources Act (Erneuerbare-Energien-Gesetz)</td>
</tr>
<tr>
<td>Em</td>
<td>Fluorescence Emission</td>
</tr>
<tr>
<td>Ex</td>
<td>Fluorescence Excitation</td>
</tr>
<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>FNBB</td>
<td>Promotion of Sustainable Biogas and Bioenergy (Fördergesellschaft für nachhaltige Biogas und Bioenergienutzung e.V.)</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward-SCattered light</td>
</tr>
<tr>
<td>GHG</td>
<td>GreenHouse Gases emissions</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
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<tr>
<td>HEL.BI.O</td>
<td>Hellenic Biogas Association</td>
</tr>
<tr>
<td>IASP</td>
<td>Institute for Agricultural and Urban Ecological Projects in Berlin Institut für Agrar- und Stadtökologische Projekte</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth medium</td>
</tr>
<tr>
<td>LP</td>
<td>LongPass filters</td>
</tr>
<tr>
<td>MW</td>
<td>MegaWatt</td>
</tr>
<tr>
<td>NawaRo</td>
<td>Nachwachsende Rohstoffe</td>
</tr>
<tr>
<td>NOx</td>
<td>Nitrogen Oxides emissions</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PAT</td>
<td>Process Analytical Technologies</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature (25°C)</td>
</tr>
<tr>
<td>SEBE</td>
<td>Sustainable and Innovative European Biogas Environment</td>
</tr>
<tr>
<td>SHMP</td>
<td>Sodium HexaMetaPhosphate</td>
</tr>
<tr>
<td>SP</td>
<td>ShortPass filters</td>
</tr>
<tr>
<td>SSC</td>
<td>Side-SCattered light</td>
</tr>
<tr>
<td>StrEG</td>
<td>Law on Electricity Feed</td>
</tr>
<tr>
<td>Syto BC</td>
<td>type of SYTO dyes</td>
</tr>
<tr>
<td>SYTO dyes</td>
<td>Fluorescent dyes for viability determination of cells in flow cytometry</td>
</tr>
<tr>
<td>Syto 9</td>
<td>type of SYTO dyes</td>
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<tr>
<td>Syto 11-16</td>
<td>types of SYTO dyes</td>
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<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TS</td>
<td>Total Solids</td>
</tr>
<tr>
<td>VFA</td>
<td>volatile fatty acids</td>
</tr>
<tr>
<td>VOC</td>
<td>Volatile Organic Compound emissions</td>
</tr>
<tr>
<td>VS</td>
<td>Volatile solids</td>
</tr>
<tr>
<td>Chemical Symbol</td>
<td>Chemical Name</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>CH₃COOH</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>CH₄</td>
<td>Methane</td>
</tr>
<tr>
<td>C₄H₈N₂O₃</td>
<td>Asparagine</td>
</tr>
<tr>
<td>C₆H₁₂O₆</td>
<td>Glucose</td>
</tr>
<tr>
<td>C₇H₇NO₂</td>
<td>p-Aminobenzoic-acid</td>
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<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>FeSO₄ · 7H₂O</td>
<td>Iron sulphate heptahydrate</td>
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<tr>
<td>H₂</td>
<td>Hydrogen</td>
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<tr>
<td>Hg</td>
<td>Mercury</td>
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<tr>
<td>H₂O</td>
<td>Water vapour</td>
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<td>H₂S</td>
<td>Hydrogen sulphide</td>
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<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Monopotassium phosphate</td>
</tr>
<tr>
<td>K₂HPO₄ · 3H₂O</td>
<td>Potassium phosphate dibasic trihydrate</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulphate</td>
</tr>
<tr>
<td>MnSO₄ · H₂O</td>
<td>Manganese sulphate monohydrate</td>
</tr>
<tr>
<td>N₂</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Disodium hydrogen phosphate</td>
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<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NH₃</td>
<td>Ammoniac</td>
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<tr>
<td>NH₄⁺</td>
<td>Ammonium</td>
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<tr>
<td>(NH₄)₂SO₄</td>
<td>Ammonium sulphate</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
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<td>Xe</td>
<td>Xenon</td>
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1. Biogas production

1.1 Biogas as an alternative sustainable energy

Over the last decades the fossil energy sources are constantly dwindling. The conservation of them and the halt of climate change are important factors for the shift to renewable energy sources. Hydroelectricity, solar energy, wind energy, wave power, geothermal energy and bioenergy are some economically competitive alternative sustainable energy sources. Bioenergy and renewable raw materials are two of the higher contributors in Germany. Renewable resources are increasingly in demand for material use, even though the growth in this area is slower than for energetic use. The sustainable way of production and use is a necessary prerequisite for the increased utilization of plant raw materials and energy sources.

Sustainability means meeting the needs of present generation without compromising the ability of future generations to meet their own needs (1987 Brundtland Report). Sustainability has three main components - economic, environmental and social. For example, the utilization of renewable raw materials needs to be balanced between the economic needs like high guaranteed biomass yields and environmental feasibility, as nature can tolerate. The social sustainability refers to new income opportunities, people’s working conditions, a share of value-added processes and other things.

Biogas is a suitable renewable energy source because it exerts a small carbon footprint in many cases. Biogas typically refers to a mixture of different gases produced by the breakdown of organic matter in the absence of oxygen. The biogas production process is further described in the following section. Agricultural waste, manure, municipal waste, plant material, sewage, green waste and food waste are some of the raw materials from which can be produced biogas.

Among the other forms of renewable energy, biogas is a versatile source, as it allows the simultaneous production of electricity and heat, as a fuel and as a natural gas substitute. Biomethane, which is methane-rich biogas, can replace natural gas as a feedstock for producing chemicals and materials. Biogas is relative easy to store and flexible in use.

From early 1990s the energy production from biogas is familiar for use in significant volume. The force of the Renewable Energy Sources Act (EEG) with some amendments in 2004 and 2009 helped to massive growth of biogas. The progress of biogas production is based on the fact that the energy generation from biogas is not
affected by the weather, time of the day or of the year and therefore is used in long
term basis in securing the basic supply of electricity known as baseload capacity. The
mitigation of greenhouse gas emissions and the conservation of the fossil energy
sources, while the energy production is lucrative is major reason which the biogas
production is spreading around the world the last decades. For example, in Germany
the biogas plants in the agricultural sector are greatly increased the last years.
Several of these plants are mono- or co-digestive energy-rich crops or products like
maize and grass silages to improve methane and energy yield.

The most important environmental advantage of biogas production is the avoidance
of additional carbon dioxide (CO₂) emissions. As opposed to fossil energy sources,
the energy production from biogas is CO₂-neutral. This means that the CO₂ released,
when the biogas is burned, was previously removed from the atmosphere during the
generation of biomass through photosynthesis. For reduce of emissions of methane
and therefore for protection of climate from damaging effects from uncontrolled
escape of methane from raw liquid manure, the manure is subjected to
fermentation. With fermentation of liquid manure the contained odours are broken
down and neutralized during the storage and thus the spreading is significantly
reduced. In addition, fermentation improves the quality of manure, as pathogens
and weed seeds are killed and nutrients made more available for plants, enabling the
manure to be applied in a more targeted fashion as a substitute for inorganic
fertilizers. Moreover, the nitrous oxide or laughing gas has an effect on the climate
and fermentation can reduce the emissions.

Figure 1 shows the variety of uses of biogas. For example, biogas is used for the
decentralized production of electricity and heat, the distribution via heat networks
and the feed-in of upgraded biogas into the natural gas grid and its following use as a
natural gas substitute for energy, as fuel or in the chemical industry.
The biogas production differs significantly in Europe. The development of use of biogas in some European countries like Austria, Sweden and Germany is rapidly as opposed to the rest of the continent and especially in Eastern Europe where there is a vast potential for this renewable energy source. This undeveloped potential is mainly due to different legal frameworks, the availability of technology, education systems and negative public perception of biogas.

The beginning of development of biogas technologies in Europe was during gas crisis in December 2008 with the first European Union project “Sustainable and Innovative European Biogas Environment” (SEBE) financed under the central program. Thereby was created an online platform to combine knowledge and launch pilot projects aimed at raising awareness among the public and developing new biogas technologies. Two months later, in February 2009, the European Biogas Association (EBA) was founded in Brussels. EBA is a non-profit organization and aims at active promotion of the deployment of sustainable biogas production and use throughout Europe created a perfect network of established national organizations (32 national organizations in 2014), scientific institutes and companies (37 companies in 2014). The three main priorities are to establish biogas as an important part of Europe’s energy mix, promote source separation of household waste to increase the gas potential, and support the production of biomethane as vehicle fuel. In 2014, the association has 70 members from 25 countries across Europe and also cooperates with biogas associations from outside Europe. EBA cooperates with the German Biogas Association (Fachverband Biogas e.V.) and Society for the Promotion of Sustainable Biogas and Bioenergy (FNBB – Fördergesellschaft für nachhaltige Biogas
und Bioenergienutzung e.V.), while the Greek EBA member is Hellenic Biogas Association (HEL.BI.O).

In Europe, Germany is the biggest biogas production and has the most advanced biogas technology. The majority of biogas plants are in Lower Saxony, Bavaria and the eastern federal states. The use of most of these plants is as power plants, which are directly connected with a combined heat and power (CHP) which produces electric power by burning biomethane. The produced electrical power is fed into public power grid.

In Germany, biogas production is carried out with co-fermentation of energy crops mixed with manure. Especially corn is the main used ‘NawaRo’ (nachwachsende Rohstoffe, which is the renewable resources). For biogas extraction organic waste and agricultural and industrial residues like food industry waste are also used.

Over the last 25 years the German legally created frameworks helped to rapidly develop the biogas production. In 1990, the Law on Electricity Feed (StrEG) was the beginning of use of renewable energies in Germany. Based on StrEG, the independent private producers of green energy could supply the public power grid and therefore the power companies had to take produced energy from them.

Apart from the advantages of biogas production there are several impacts facing the management and organization of renewable energy supply because of rapid development. One of these impacts is the high area-consuming of the biogas electric power supply. In Germany over the last years a big area is used for energy crops. This demand of agricultural areas generates new competitions with the food industries. Therefore, new industries and markets were created in rural regions and were acting to gain all offering advantages of this new energy source. These new players have economic, political and civil dimension. With government focused in these acts, biogas will continue to play an important role in the German renewable energy supply.

According to German Biogas Association, the biogas segment statistics of development of the number of biogas plants and the total installed electric output in megawatt (MW) in Germany as of November 2014 are shown in Figure below.
German Biogas Association provides also a table with biogas sector statistics which includes the number of biogas plants, the installed electric capacity, the gross electricity production, the number of households supplied with biogas-based electricity, the CO2 reduction by biogas, the turnover in Germany and the jobs in the biogas sector. (Table 1)

Table 1. Biogas Sector Statistics in Germany at a Glance as of November 2014 (Source: German Biogas Association, Fachverband Biogas e.V., 2014)

<table>
<thead>
<tr>
<th></th>
<th>2013*</th>
<th>Forecast 2014**</th>
<th>Forecast 2015**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of biogas plants</td>
<td>7,850 (144)</td>
<td>7,944 (153)</td>
<td>8,005 (155)</td>
</tr>
<tr>
<td>(biogas plants with biogas plants injection)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Installed electric capacity in MW</td>
<td>3,543</td>
<td>3,859</td>
<td>4,054</td>
</tr>
<tr>
<td>Gross electricity production in TWh per year</td>
<td>26,42</td>
<td>27,55</td>
<td>27,88</td>
</tr>
<tr>
<td>Households supplied with biogas-based electricity in millions</td>
<td>7,5</td>
<td>7,9</td>
<td>8,0</td>
</tr>
<tr>
<td>CO2 reduction by biogas in million tons</td>
<td>16,8</td>
<td>17,6</td>
<td>17,8</td>
</tr>
<tr>
<td>Turnover in Germany in Euro</td>
<td>7,3 Billion</td>
<td>7,4 Billion</td>
<td>7,2 Billion</td>
</tr>
<tr>
<td>Jobs in the biogas sector</td>
<td>41,000</td>
<td>41,000</td>
<td>39,000</td>
</tr>
</tbody>
</table>

* Own extrapolation based on country data
** Based on an expert survey
In Greece the biogas production has not been developed to the extent that has been developed in Germany. The Hellenic Biogas Association (HEL.BI.O) is the first non-profit organization in Greece. It was founded to promote anaerobic digestion to produce biogas and fertilizer. The Association's members consist of eminent scientists of the biogas sector, entrepreneurs with investments in biogas plants and companies. The objective of Hellenic Biogas Association is to become the official body of the biogas sector, helping and supporting all efforts, which will help develop the industry, highlighting the unique advantages of both Greek economy and environmental protection.

The Hellenic Biogas Association (HEL.BI.O), member of European Biogas Association, was founded to promote the anaerobic digestion of all kinds of organic substances, organic waste and agro-industrial waste to produce biogas and fertilizer in a sustainable manner. The study and application of best available technologies for integrated management and utilization of solid waste and biomass in general and simultaneously the use of biogas, fertilizer and any other product produced by the anaerobic digestion in Greece are major priorities of the company. The HEL.BI.O aims to the optimal design, the construction and the management of biogas plants, the provision of advisory services on biogas projects and also coordinates the action of partners-members to achieve their common goals and harmonious cooperation and mutual support between them.
1.2 The anaerobic fermentation for methane synthesis

Biogas is a gas product of methanogenic bacteria decomposition of organic materials in anaerobic fermentation, which could be a moist environment under exclusion of oxygen. These conditions are ideal for almost entire conversion of organic matter into biogas. The decomposition, so-called also anaerobic digestion, takes place in marshes and wetlands, on the bottom of lakes, in swamps and also in the digestive tract of ruminants.

The anaerobic transformation of organic wastes is a microbial process, which includes the complex interaction of various microorganisms, such as hydrolysing, acidifying, acetogenic and methanogenic bacteria, which are responsible for the methane ($\text{CH}_4$) and carbon dioxide ($\text{CO}_2$) production, the main products of the digestion process. The biogas process takes place in four basic separate phases, the hydrolysis phase, the acidogenesis stage, the acetogenesis step and the final phase of methanogenesis.

During the first phase of biogas production, which is the hydrolysis of the polymerized, mostly insoluble organic compounds as are the carbohydrates, the proteins, the nucleic acids and the fats, soluble monomers and dimers, that is monosugars, amino acids and fatty acids are produced though bacteria action.

In this stage, extracellular enzymes from the group of hydrolases, which involves the amylases, proteases and lipases, produced by appropriate strains of hydrolyzing bacteria ($\text{Bacterides, Clostridia}$ etc.) help the decay of complex organic macromolecular compounds. Cellulose and cellucotton are hardly decomposable polymers and their hydrolysis reduces the rate of wastes digestion. According to Parawira et al., 2008, the half amount of solid organic wastes undergoes biodegradation while the rest part of compounds remains in their primary state because of the lack of enzymes participating in their degradation. Hydrolysis is a crucial step because it determines the amount of produced methane. The rate of process depends on various parameters, such as pH, size of particles, production of enzymes and diffusion and adsorption of enzymes on the particles of wastes subjected to the digestion process.

In the second step, in the acidogenesis, the products of hydrolysis, which are watersoluble chemical substances, are converted to short-chain organic acids as are formic, acetic, propionic butyric and pentanoic acid, alcohols as are methanol and ethanol, aldehydes, carbon dioxide and hydrogen by acidifying bacteria.

The commonly products are the acetic acid ($\text{CH}_3\text{COOH}$) by 50%, the carbon dioxide ($\text{CO}_2$) and the hydrogen ($\text{H}_2$) by 20% and the volatile fatty acids (VFA's) and the alcohols by 30%. Acetic acid ($\text{CH}_3\text{COOH}$), carbon dioxide ($\text{CO}_2$) and hydrogen ($\text{H}_2$) are
methanogenic substrates and can be directly converted to methane (CH₄). Instead, fatty acids and alcohols may not be used directly by methanogenic bacteria and have to be converted by obligatory bacteria producing hydrogen in the next stage of acetogenesis. From decomposition of proteins, amino acids and peptides could be an energy source for anaerobic microorganisms. Moreover, the produced ammonia and hydrogen have an intensive unpleasant smell.

In acetogenesis, the acetate bacteria, like the genera of *Syntrophomonas* and *Syntrophobacter*, convert the acid phase products into acetate and hydrogen, which act as a substrate for methanogenic microorganisms. The decomposition of pentanoic acid to propionic acid helps bacteria *Methanobacterium suboxydans* while *Methanobacterium propionicum* helps the decay of propionic acid to acetic acid. The produced hydrogen is released causing toxic effects on acetogenic microorganisms. In this stage, the volatile fatty acids and alcohols are oxidized to acetic acid, hydrogen and carbon dioxide. In acetogenesis phase the efficiency of biogas production is determined since approximately 70% of methane arises in the process of acetates reduction which is the main intermediate product of the process of methane digestion. Apart from methane, in this stage approximately 25% of acetates and 11% of hydrogen are formed.

In the final phase, methanogenesis, the production of methane is based on methanogenic bacteria action. The products of the previous stages as are acetic acid, H₂, CO₂, formate, methanol, methylamine and dimethyl sulfide are the substrates for methane production. According to Demirel and Scherer, 2008 and despite the fact that there are only few bacteria which could convert acetic acid into methane, the majority of methane arising in the methane digestion process is the result of heterotrophic methane bacteria action. The results of CO₂ reduction by autotrophic methane bacteria is 30% of methane production in the process. The depletion of hydrogen creates good conditions for the development of acid bacteria which cause short-chain organic acids in second phase (acidogenesis) and therefore too low H₂ production in acetogenic phase. If there is a gas rich in CO₂ after conversion, then it is hard to be converted into methane. The last phase is the critical step of the whole process, as is the slowest and rate controlling.

Figure 3 below shows simply the process of biogas production.
Figure 3. Simplified diagram of the organic materials decomposition during biogas production. (Source: Fachagentur Nachwachsende Rohstoffe e. V.)

Table 2 depicts the main components of final gas mixture formed during biogas production.

Table 2. Main components of final gas mixture formed during biogas production

<table>
<thead>
<tr>
<th>Component</th>
<th>Formula</th>
<th>Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methane</td>
<td>CH₄</td>
<td>50-75</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>CO₂</td>
<td>25-45</td>
</tr>
<tr>
<td>Water vapour</td>
<td>H₂O</td>
<td>2-7</td>
</tr>
<tr>
<td>Hydrogen sulphide</td>
<td>H₂S</td>
<td>0.002-2</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>N₂</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Oxygen</td>
<td>O₂</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Ammoniac</td>
<td>NH₃</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>H₂</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Trace gases</td>
<td></td>
<td>&lt; 2</td>
</tr>
</tbody>
</table>
The type of used raw material and the method of conducting this process determine the composition of final products and especially methane. The methane content in final gas mixture is closely linked to the energy content of the biogas. Fats and starch are substances, which could be easy converted and consequently the high content of them equivalent to the great gas yield. Because the four mentioned phases of the process take place simultaneously, it is necessary to find a suitable environment for the various microorganisms combining the most important parameters such as temperature, pH and supply of nutrients.

The anaerobic digestion process is very sensitive to disturbances, which can emerge due to technical reasons or to inhibitors. Even in small quantities of inhibitors the decomposition process and specially bacteria could have harmful effects. Inhibitors enter the fermenter with two possible ways; with the substrate or with intermediate products of the decomposition process. An excess amount of substrate in the fermenter could adversely affect the bacteria and therefore inhibit the fermentation process.

Some substances as are disinfectants, detergents, antibiotics, solvents, herbicides, salts and heavy metals, are also not conducive to the biogas production. High concentration of these substances will be poisonous to microorganisms. Other product of fermentation process, which is toxic to cells and inhibits the decomposition is the hydrogen sulphide. However, sulphur is an essential mineral trace element for methanogenic bacteria. Finally, ammonia is a main inhibitor of methane production when presents at high concentration and therefore the poultry droppings pig liquid manure are diluted or mixed with low nitrogen substrates.

A great amount of organic substrates can be used for biogas production. In agricultural biogas plants, animal excrements like cattle and pig liquid manure and energy crops, plants grown for energy production are the commonly used substrates. Energy crops help to be made new biomass each year to produce electricity, heat and fuel. Other organic wastes from the processing industry and municipalities and agriculture and industry residues can also be fermented for biogas production. The utilization of available slurry and other farm fertilizers helps to climate protection avoiding the harmful emissions.

Renewable raw materials are maize, grain, grass, cereal crops, millet, sunflowers and sugar beets. Maize has highest use among the renewable resources because it offers high dry matter yields and energy yields requiring less fertilizer and plant-protection products and consequently offers the best efficiency, technological suitability, and cost structure. The only negative effects of maize are the influence on soil fertility and biodiversity.

Apart from renewable raw materials, other suitable substrates are excrements, feed remains and other agricultural and non-agricultural residues. Especially, these are
residues from the food industry such as pomace, vinace, distiller’s wash and grease separator residues, vegetable waste from wholesale markets, food waste from restaurants, grass clippings, material from landscape conservation, or organic waste from municipal waste disposal.

Figure 4 depicts the variety of substrates with the methane content and the fluctuations in the biogas yield. The installation technology of the biogas plant, the potential of substrates for gas formation and biological key figures of the fermentation process have a major signification to gas output content. The daily generated energy content from biogas plant is approximately 6 kWh/m³ of biogas.

![Figure 4. Methane content and biogas yield of various substrates (KTBL, 2010; FNR, 2013).](image)

The anaerobic digestion offers significant benefits to biogas production compared to other types of bioenergy production. According to Fehrenbach et al., 2008, the anaerobic digestion is an energy efficient and environmental beneficial technology for the production of bioenergy. Some of direct environmental advantages are the
reduction of greenhouse gases (GHG) emissions compared to fossil fuels by utilization of locally available resources and the potential reduction of Volatile Organic Compound (VOC) emissions. With anaerobic digestion control of odor and of pathogen and weed seed is observed. The digestate is an improved fertilizer in terms of its availability to crops which can substitute mineral fertilizer. The only potentially environmental impact of anaerobic digesters combusting the biogas is the nitrogen oxides (NOx) emissions. Nitrogen oxides are main air pollutants and the precursor of ozone.

The effectiveness of anaerobic digestion of different waste composition and efficiency of the anaerobic digester is judged by five criteria which are the methane production rate, the degree of reduction of the organic load, the stability of microbial activity, the amount of produced biogas and finally the economic viability of the process. These criteria are influenced by parameters which interact with each other as are the temperature, the hydraulic residence time, the pH, the concentration of volatile fatty acids, the various nutrients and the toxicity. These factors are carefully monitored as indicated the proper and the optimal operation of anaerobic digesters.
1.3 Typical process analytical tools applied at biogas plants

In 2005, the United States Food and Drug Administration (FDA) defined a guide for industry in order to facilitate continuous process improvement and optimization of productivity and product quality. The appropriate measurement program and technologies which design, analyze and control manufacturing processes was defined the Process Analytical Technologies (PAT). In the beginning, this innovate system was applied in the pharmaceutical fermentation and processing industry, but it is prevalent in various industries nowadays. The term 'analytical technology' in PAT refers to analytical chemical, physical, microbiological, mathematical, data and risk analysis conducted in an integrated manner while the term 'quality' of a product refers the final quality of various industrial processes.

The desired goal of PAT is the design and development of processes, which could consistently ensure a predefined quality at the end of the manufacturing process. These processes manage the variability and simultaneously all critical sources of variability are identified and subsequently explained. When the product-quality attributes can be accurately and reliably predicted over the design space established for materials used, process conditions, manufacturing, environmental, and other conditions, the process is considered well understood and the goal of PAT is gained.

As shown in Figure 5, PAT can be depicted as a three-step process. The first step of the process development is the design of unit operation, the optimization and ultimately the characterization of the unit. This step is considered critical for the next two phases of PAT because in the phase of design, the critical process parameters (CPP) are identified along with the critical quality attributes (CQA). The critical quality attributes (CQA) are being affected by the process step while the critical process parameters (CPP) affect the critical quality attributes (CQA).

In the next phase, in the analyze phase, the critical quality attributes (CQA) and the critical process parameters (CPP) are monitored by a suitable analyzer. The at-line PAT application involves the remove, the isolation from and the analysis of sample close to process stream. The on-line PAT application comprises the sample remove for analysis from process stream and the return to stream. A further PAT application is in-line in which the sample is not removed, but it is analyzed in place. PAT application can be also off-line when the sample is removed and analyzed away from process stream. For all kind of PAT application, it is necessary the analytical results to be available on time in order to facilitate the real-time decision making.

The last but highly significant step is the control step. It includes the design of an appropriate control scheme based on the process understanding. The obtained data
can be utilized for making real-time process decisions, and therefore consistent process performance and product quality can be achieved.

![Three-step PAT implementation process](image)

Figure 5. Three-step PAT implementation process. CQA is the abbreviation of critical quality attributes and CPP of critical process parameters.

Because of the great number of biogas plants in Europe a significant interest in online monitoring and control is created over the last years and consequently a substantial research for appropriate instrumentation, control, and automation of anaerobic processes is in progress. These current investigations are faced with the practical and preferable non-expensive optimization of the partial stages in the biogas plant such as the improvement of biogas production. These current investigations are faced with the practical and preferable non-expensive optimization of the partial stages in the biogas plant such as the improvement of biogas production. However, there are some complex and multi-faceted problems in anaerobic digestion systems like problems reducing CO$_2$ and H$_2$S fractions while increasing CH$_4$, dealing with biogas composition and coupled with increasing biogas production volume.

For efficient control decisions and optimization of productivity it is better to have the appropriate process information regarding the anaerobic digester process such as information about biomass, substrates, intermediates, digestate and nutrients. These information help also the decrease of the variability and thus probably to improve the product, biogas quality. Optimizing efficiently the anaerobic digester
process the various substrates is properly utilized as well as high biogas yields is demanded.

As mentioned above the anaerobic digestion of organic materials is a highly complex, multi-stage process and is influenced by a great number of microbiological, chemical and physical factors and consequently the recording of diverse indicators is required. The individual stages of decomposition of biogas production take place at different speeds and the process control becomes more complicated. Therefore, the dynamic behavior of the process depends to a great extent on the material composition of the used substrates.

Many biogas plants do not continuously measure and utilize in-depth process data when measured. In some cases, the measurements are done for routine monitoring and control parameters and not for real-time process control. The easily measurable parameters as are temperature, pH value and biogas production and volume, are daily evaluated. The values of total suspended solids, volatile organic acids, and ammonia nitrogen are measured at regular intervals like monthly off-line measurements. The number of measurements is often limited due to fact that the costs of analysis are high and there is lack of trained personnel. However, there are some less expensive methods for laboratory analysis, but these measurements cannot give a fast response to facilitate process imbalances during the anaerobic digestion process. An example of these developments is fast photometric tests for ammonia nitrogen and fatty acids.

It is imperative for on-line and continuous monitoring of the fermentation to be developed more robust and sophisticated techniques. Using these tools the control of process will be better, the stability will be increased and the fermentation process will be run at optimal conditions. The result of utilization of these techniques is the economy increase of biogas plants. The increasing economic and technical operational targets require the process optimization through applying advanced on-line monitoring. Identifying the chemical and physical parameters, an early warning of the process imbalances will be given and thus the improvement of the plant efficiency is achieved. The monitoring tools are technically simple and require little attention and maintenance in order to be of interest for the biogas plants. According to Moletta et al., 1994, the main criteria for system selection are three. The minimum number of sensors for a wide range of parameters and the availability of reliable sensors for the chosen measurements are the two prerequisites. The third one is that the response time of the sensors measuring chosen parameters should be adequate to the response time of the process phase and measurements in real time.
Anaerobic digestion can be monitored in different process phases. Firstly, it can monitor the quality of the substrate and its potential measured the total solids (TS) and/or the volatile solids (VS) or the demand of chemical oxygen (COD) and their degradation during the process. Through influence of intermediates products formation as are volatile fatty acids (VFA), hydrogen ($H_2$), carbon monoxide (CO) and ammonium ($NH_4^+$) can monitor the alkalinity and pH values. The overall biogas production or the ratio of methane ($CH_4$) to carbon dioxide ($CO_2$), $CH_4/CO_2$ rate, can monitor the formation of final product. Microbial communities and their activities can also be a measuring tool in biological processes.

The temperature and the pH are main compulsory measurements in biogas plants. These are measured in real-time at biogas plants to ensure a suitable environment for all bacteria. It is usually used simple electronic pH meters and temperature sensors embedded in the digester. It is important to be maintained a constant temperature during the anaerobic digestion process because the temperature affects strongly the process from a kinetic and thermodynamic point of view.

From the production point of view the major measurement is the biogas volume. Methane is the valuable product from biogas production and consequently overall biogas production or methane yield is measured. The most common expression of yield is $Nm^3$ biogas/t feed. The methane production is expressed as $Nm^3 CH_4/day$ or $NmL CH_4/gVS$. Measuring the produced biogas there is not any information about the previous phases, but only the final product. If the overall biogas production or the methane yield is low that means that the fermentation was disturbed. Information about the process stability and efficiency is given by measuring of gas composition. The ratio of methane ($CH_4$) to carbon dioxide ($CO_2$) is usually stable during the process and any change means also that the fermentation was disturbed. This disturbance is justified because this ratio depends on factors as are the quality of substrate, the temperature of process and the pH value. Therefore, the methane yield provides more valuable and reliable information than the biogas ratio.

In this study, Electrooptical measurements are used as a process analytical tool. The results are compared with the results from Flow Cytometry, another recent developing analytical technology.
2. Flow Cytometry

2.1 Introduction to Flow Cytometry

Flow Cytometry is a laser-based technique used in biotechnology to assess biological and physiological characteristics of several types of cells. Most applications of flow cytometry are witnessed in human cells, while the analyses of bacterial cells have just recently been under observation. This technology can be employed as an at-line monitoring tool in bioprocess development, for example in microbial biofuels production processes (Silva, Roseiro et al. 2012). It is widely used as a method to simultaneously quantify cellular characteristics and the levels of cellular components. Assays that employ flow cytometric techniques have been established to determine both cellular characteristics and the constituents of cellular components such as DNA, protein, surface receptors, and calcium. A flow cytometer is a combined system of fluidics, optics and electronics. The objectivity, high analytic performance rate, precision and high sensitivity are the reasons for the implementation as a process analytical tool. Basically, a flow cytometer composed of the fluidics system which is responsible for confinement of cells for individual analysis in laser beam. The optics system constitutes the source for the lasers which illuminate the cells in the cell suspension and as well as optical filters which direct the resulting light signals to the appropriate detectors. Finally, the electronics system converts the optical signals into electronic signals, which can be processed by the computer using appropriate software. In contrast to the conventional microbiology methods, it is possible, using several staining dyes, to differentiate cells based on their structure and metabolic activity.
2.2 Principle and Mode of Operation

The diagram in Figure 6 depicts a typical flow cytometer and how the three main systems are linked.

![Diagram of a flow cytometer](image)

**Figure 6.** The combination of the systems in a flow cytometer. The fluidics system restraints the cells in separate confinements so that the laser beam can analyze them individually. Subsequently, the optical signals that go through the dichroic filters and mirrors are detected by appropriate detectors (FS, SS, FL1, FL2, FL3). The electronics system, finally, converts these signals to electrons which can be processed by a suitable software (Source: Díaz, et al. 2010).

The fluidics system consists of the central core enclosed by the outer sheath. In the central core, the cell suspension is injected and the particles are randomly distributed. As the sheath fluid moves at faster rate than the cell suspension, it creates a drag effect on the narrow central chamber, resulting in velocity variation of the central fluid. The flow profile turns out parabolic with greatest velocity at the center and zero velocity at the wall. The sample pressure is always greater than the sheath fluid pressure. Because of the pressure differences and optimal conditions associated with the laminar flow, the cell suspension and the sheath fluid are not mixed. The effect called hydrodynamic focusing forces cells to pass in single file through the light source. Without hydrodynamic focusing it would not be possible to analyze one cell at time because the nozzle of the instrument would be blocked (Rahman, 2006).

After the hydrodynamic focusing, each cell passes through one or more beams of light and the signals emanating from each cell are channeled to the electronic
system for further processing. As opposed to the other systems which used arc lamps such as the Xenon (Xe) and Mercury (Hg), the laser implicated in the current systems possess distinct advantages. The fact that lasers produce light beams of the same wavelength, with different frequencies constitutes advantageous properties that promote its use in analyzing samples. Nevertheless, arc lamp which simply exploits the color of an ignited gas in a sealed tube is cheaper. However, the production of unstable scrambled light with a mixture of wavelengths limits its application in online analysis since the incoherent light requires subsequent optical filtration (Rahman, 2006). Therefore, the main difference between lasers and arc lamps is attributed to their mode or field of application. The selection of the laser will depend on the type of cell analysis and on the range of wavelengths required for the excitation of the selected fluorescent markers.

The deflection of the incident laser beam by a particle results in light scattering as demonstrated in Figure 7. The extent of this scattering depends on the physical properties of a particle, the size and the internal complexity. Some of the factors that affect light scattering are the cell’s membrane, nucleus, cell shape and surface topography (Biosciences, 2000). There are two types of scattering to detect the cells. Forward-scattered light (FSC) is one of the types which is measured in the plane of the laser light source (0-10°) and the second type is the side-scattered light (SSC), which is measured at 90° to the laser beam. FSC provides information on cell size while SSC is related to cell granularity and internal complexity. Because scattering signals have a characteristic profile for each particle, they can distinguish the different cell types in heterogeneous samples like biogas samples.

The optics system is formed by several dichroic mirrors and filters which absorb certain wavelengths, while transmitting others towards the proper detectors. The detectors are photodiodes or photomultiplier tubes which receive the signals for subsequent analysis. There are three types of filters: longpass, shortpass and bandpass filters. Longpass (LP) filters allow light to traverse above a cut-off wavelength while shortpass (SP) filters permit light below a cut-off wavelength.
Lastly, bandpass (BP) filters just transmit narrow band width light as shown in Figure 8.

Figure 8. Longpass, shortpass, and bandpass filters and their response. (Source: Biosciences, 2000)

Subsequently, detectors measure the magnitude of a pulse which represents the extent of light scattered or fluorescence. The photomultiplier tubes are more sensitive and appropriate than the photodiodes for the detection of scattering and fluorescence signals. A flow cytometer can measure scattering signals and several fluorescence parameters depending on the number of lasers and detectors which are available every time. Finally, the electronics system converts the optical signals into electronic signals, which can be processed by the computer using appropriate software.
2.3 Dyes typically applied for biogas samples

Fluorochromatic reagents that have found plausible applications in flow cytometry are dyes with specific properties. These dyes accept light energy at a specific designated wavelength and re-emit this energy at a longer wavelength. These two processes are called excitation and emission respectively. The process of emission is referred to as fluorescence and it is extremely rapid. The wavelength for maximal absorption and emission determines the optimal operation conditions for a certain dye.

Dyes used in flow cytometry require distinctive properties, particularly their inertness to the biological system as well as being able to demonstrate a high extinction coefficients and high quantum yield. These two properties ensure that small concentrations of the stain can produce a significant signal within the cell. Since only a single cell is analyzed at a time, this helps to prevent overlapping and ensure photostability, low toxicity as well as solubility of the dye in water within the narrow emission spectrum (Díaz, et al. 2010).

There is a wide range and extensive variety of dyes used in combination with flow cytometry. Table 3 shows some available dyes, and their excitation and emission maximal wavelengths.
### Table 3. Some of basics dyes used in flow cytometry. *Fluorescence excitation (Ex) and emission (Em) maxima in nm.*

<table>
<thead>
<tr>
<th>Cell ligand or substrate</th>
<th>Dye</th>
<th>Ex/Em&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA, RNA</td>
<td>Ethidium bromide (EB)</td>
<td>510/595</td>
</tr>
<tr>
<td></td>
<td>Propidium iodide (PI)</td>
<td>536/623</td>
</tr>
<tr>
<td>Cyanines</td>
<td>TO-PRO 515/533 (TO-PRO 1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>462/661 (TO-PRO 3)</td>
</tr>
<tr>
<td></td>
<td>TOTO 514/533 (TOTO 1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>642/660 (TOTO 3)</td>
</tr>
<tr>
<td></td>
<td>SYTO 485-508/498-527</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SYBR 497/520 (Sybr Green)</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>DAPI</td>
<td>350/470</td>
</tr>
<tr>
<td></td>
<td>Hoechst 33342</td>
<td>350/461</td>
</tr>
<tr>
<td></td>
<td>DRAQ5</td>
<td>647/700</td>
</tr>
<tr>
<td>Total protein</td>
<td>FITC</td>
<td>495/525</td>
</tr>
<tr>
<td>Lipids</td>
<td>Nile Red</td>
<td>551/636</td>
</tr>
<tr>
<td>Fluoresogenic substrates</td>
<td>ChenChrom dyes (CY, CB, CV6)</td>
<td>488/520</td>
</tr>
<tr>
<td></td>
<td>FDA, CFDA, CFDA/SE, CFDA-AM</td>
<td>492/519</td>
</tr>
<tr>
<td></td>
<td>Calcein-AM</td>
<td>494/517</td>
</tr>
<tr>
<td></td>
<td>CTC</td>
<td>450/630</td>
</tr>
<tr>
<td></td>
<td>FUN-1</td>
<td>480/580</td>
</tr>
<tr>
<td>Intracellular pH</td>
<td>BCECF-AM</td>
<td>482/520</td>
</tr>
<tr>
<td></td>
<td>SNARF-1</td>
<td>510–580/587–635</td>
</tr>
<tr>
<td>Membrane energization</td>
<td>Rh-123</td>
<td>507/529</td>
</tr>
<tr>
<td></td>
<td>DiOC&lt;sub&gt;6&lt;/sub&gt;(3)</td>
<td>484/501</td>
</tr>
<tr>
<td></td>
<td>DiOC&lt;sub&gt;2&lt;/sub&gt;(3)</td>
<td>482/497</td>
</tr>
<tr>
<td></td>
<td>DiBAC&lt;sub&gt;6&lt;/sub&gt;(3)</td>
<td>488/525</td>
</tr>
<tr>
<td></td>
<td>JC-1</td>
<td>498,593 / 525,585</td>
</tr>
<tr>
<td>Citoplasmic Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Indo-1</td>
<td>361,330/405, 480</td>
</tr>
<tr>
<td>Antibodies or oligonucleotides</td>
<td>BODIPY</td>
<td>503/512</td>
</tr>
<tr>
<td></td>
<td>PE, PE-Cy5 conjugates</td>
<td>490/575, 690</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor 488</td>
<td>495/519</td>
</tr>
<tr>
<td></td>
<td>Oregon green</td>
<td>496/524</td>
</tr>
<tr>
<td>Autofluorescent proteins</td>
<td>GFP and derivatives</td>
<td>489/508</td>
</tr>
</tbody>
</table>

Fluorochromes have different mode in relation to the intracellular and extracellular properties. Some dyes interact specifically to cellular molecules or components (nucleic acids, proteins and lipids). These interactions lead to an increased fluorescence of these individual molecules. Others act differently by accumulating selectively in cell compartments, or cellular environmental conditions such as pH,
membrane polarization, membrane integrity, enzymatic activity and respiratory activity which might cause the dyes to modify their properties through specific biochemical reactions (Figure 9) (Díaz, et al. 2010).

**Figure 9.** Fluorescence stains applied in flow cytometry and their function. All stains have to pass through the cytoplasmic membrane to be detected by flow cytometry. Functions: Syto 13, Propidium Iodide (PI) and DAPI for membrane integrity; DiBAC4 (BOX) for membrane potential. (Source: Díaz, et al. 2010)

**Propidium iodide (PI)**

Propidium iodide (PI) is the most commonly used dye to quantitatively assess DNA content. While in microscopy PI is used to visualize the nucleus of cells and other DNA containing organelles, in flow cytometry it is used to evaluate cell viability or DNA content in cell cycle analysis. PI can enter cells, which have lost their membrane integrity and stain the DNA. Therefore it is used to evaluate the cellular viability.

The mechanism of action of PI involves binding to DNA by intercalating between the bases with little or no sequence preference. The stoichiometry of this binding is one dye per 4–5 base pairs of DNA. However, PI also binds to RNA, which is why samples should be treated with nuclease to distinguish between RNA and DNA staining. This dye has an absorption maximum at 536 nm and emission maximum at 623 nm. Excitation energy can be supplied with a xenon or mercury-arc lamp or with an argon-ion laser (488nm).
4′6-diamidino-2-phenylindole (DAPI)

The widest application of 4′,6-diamidino-2-phenylindole (DAPI) is in flow cytometry. DAPI is a popular nuclear counterstain which can be used alone or in multi-parameter cell analysis in combination with another fluorochrome. It is also used extensively in fluorescence microscopy.

The blue-fluorescent DAPI is a nucleic acid stain binds strongly to A-T rich regions in DNA. Thus, the resulting fluorescence depends not only on the amount of DNA in the cell, but also on A-T base content of the DNA. DAPI can pass through an intact cell membrane and therefore can be used to stain both live and fixed cells. It passes through the membrane less efficiently in live cells. The maximum absorption of this dye is at 358 nm and the maximum emission is at 461 nm.

Bis-(1,3-dibutylarbituric acid)-trimethine oxonol (BOX)

There several terminologies used to refer to bis-(1,3-dibutylarbituric acid)-trimethine oxonol such as; diBAC4 (3) (Deere, Porter et al. 1995). BOX (Want, Thomas et al. 2009), BOX, bis-oxonol, BOX, and DiBAC4 (Hewitt and Nebe-Von-Caron 2004). In this study, the term used is BOX.

BOX is mainly used to evaluate the membrane potential or in other words to check the vitality of cells. It is impermeable to a polarized membrane due to its anionic charge. Nevertheless, when cells lose their membrane potential total or partially, BOX can passively diffuse through the cell membrane. BOX binds to positively charged proteins or unspecifically to hydrophobic regions, but not to the cell wall or outer membrane structures of living cells. This lipophilic dye has an excitation maximum of 488 nm and emission maximum of 525 nm.

SYTO 13

SYTO dyes are cell-permeant nucleic acid stains. These dyes can be used to stain DNA and RNA in both live and dead eukaryotic cells, in gram-positive and gram-negative bacteria. There are a variety of SYTO stains, such as Syto 9, Syto 11-16 or Syto BC. These dyes are permeable to bacterial membranes and mammalian cells. They have high molar absorptivity and also extremely low intrinsic fluorescence which is attributed to their low quantum yields (typically less than 0.01) in the ‘free’ state, which consequently increases above 0.4 when bound to nucleic acids. The SYTO dyes do not act exclusively as nuclear stains in live cells and should not be equated with DNA-selective compounds such as DAPI, which stain nuclei in live animal cells. Excitation energy can be supplied with a xenon or mercury-arc lamp or with an argon-ion laser.
In this study, SYTO 13 is used. SYTO 13 stains both DNA and RNA. The maximum excitation of DNA is at 488 nm and the emission maximum at 509 nm. The excitation peak maximum for RNA is 491 nm and the emission peak maximum is 514 nm. For SYTO 13, excitation energy can be supplied with an argon-ion laser which is available in the most flow cytometers.
3. Electrooptical detection of the cell polarizability

EloTrace is the first world-wide automatic electrooptical measurement system for detection of parameters of bacterial cells. The continuous electrooptical monitoring uses high precision optical sensors. The induced polarized charges at cellular membranes are quantified by the sensors and the level of the polarizability is closed linked to the actual physiological state of the cell. The system does not use any reagents, chemicals or other consumables and is therefore very affordable in terms of running costs.

The main fields of application of EloTrace are the production of starter cultures, bacterial protection cultures, probiotic cultures, recombinant proteins or other biosubstances such as recombinant human insulin and interferone, biological substances like technical enzymes, fine chemicals, aminoacids, new materials and production and quality control of the manufacturing of bacterial vaccines.

The monitoring of biosynthesis activity, the optimization of fermentation conditions as well as the controlling is the most important application of EloTrace. The process control achieves a balance growth and improves understanding about growth behavior and critical phases of a fermentation process. The high degree of automation helps to determine the ideal time for harvesting with a maximize yield of stable active cells as well as increase of product quantity and quality of fermentation runs.

Using EloTrace, the determination of cell activity, stress levels, cell size and morphological changes is done automatically. Therefore, the observation of division frequency is immediately and it is easy to predict the culture development and the points in time with higher cell vitality. Based on these results, it is possible to choose the optimal time points of inoculation, induction and harvest. Additionally, the device uses high number of cells for statistical proposes.

The employed electrooptical measurement technology 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 have a polarizing effect on the ions in the cell cytoplasm, which are unable to leave the cell through the membrane due to the membrane’s electrophysiological properties. Therefore the charged ions are concentrated near the membrane due to their effort to orient themselves in the electrical field. This in turn leads to a minuscule force affecting 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 precisely picked up by extremely sensitive high-end photometric sensors (Biotronix) (Figure 10).

![Diagram](image.jpg)

**Figure 10. Electrooptical measurement technology of EloTrace.**

The diagram in Figure 11 depicts the whole sample preparation process by the results shown on the computer. Before measurement, the sample is prepared properly. The cells are separated and washed once to determine a good reproducibility and conductivity. After this step the cells are resuspended in water till to adjust the optical density (OD) to 0.1. The cells are ready and moved to the electrical chamber. The electrical field redirects cells in the correct position to be measured. The photo detector is measuring continuous the OD of cells and simultaneously the needed time to redirect the cells is a direct measure of the transmembrane potential. So, the polarizability of the cells is known. Different frequencies of electrical field can be applied.
Figure 11. Operation process of EloTrace. Firstly, the cells are separated and washed with water. The second step is to resuspend the cells and adjust the OD to 0.1. Subsequently, the sample enters to the electrical field and the photo detector is measuring the OD and the needed time to redirect the cells. Using intelligent software the results are processed and showed in various graphs.

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. One of the main parameters is the Anisotropic Polarizability value (AP) which is the degree of polarizability of electrical charges in both cytoplasm and membrane and reflects directly the ions distribution on cell structures and intracellular ionic pool. This value also leads to information about transport activity, energy level of membrane, intracellular metabolic flux and inhibition/stress level. Other main parameters are the cell size/length of bacteria with the typical elongated cell morphology, the biomass concentration which is calculated by optical density measurement and cell concentration.

The physical measurement principle of AP is based on „Maxwell-Wagner Effect” namely on the polarizability of electrical charges/ions at the intact cell membrane. The level of AP value is related to the change of the geometrical orientation of the bacteria cell within the suspension as it reacts to the created electrical current field during the measurement. The free ions and charged metabolic intermediates within the cytoplasm of the cell participate in the energy status as well as in intracellular transport activity of the cell membrane. The concentration of these mobile ions is directly correlated with AP level. The higher AP values the more stable growth and high productivity of a cell or cultivation. The adverse pH-levels, a decrease in O₂ or
substrate uptake, damage or “depolarization” of the cell membrane are some of negative conditions and the result of them is the decrease of the values.

The combination of the full automation of the measurement and the high sensitivity of the employed measurement technology with the knowledge how elongated particles “relax” in liquid environments leads to high 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.

Additionally, the biomass control is done automatically on-line using the values of high cell density fermentation. The determination of the cell concentrations (cell/mL) is based on the determined OD-value and the measured cell size.

Finally, the EloTrace is extensively used for process control because of on-line measurement which is ideal for process documentation and scale-up. The knowledge about and understanding of growth behavior as well as critical phases in the fermentation development are greatly increased with this automatic system. The detection of the occurrence of „overflow metabolism“ is also 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. One of the main benefits of EloTrace is that the determination of the perfect time for harvesting leading to an optimized yield of stable and active cells is done with unprecedented precision.
Materials and Methods

1. Cultivation of Clostridium acetobutylicum ATCC 824

*C. acetobutylicum* is a proteolytic gram-positive bacillus. It has been found in a number of different environments, most notably in soils, but also in well water, clam gut, lake sediments and isolated from canine, bovine and human feces (Cato et al., 1986). This bacterium is rod-shaped, motile by peritrichous flagella, and produces subterminal endospores. The range of optimal temperatures, which all the strains of *Clostridia* grow, is 10-65°C. According to Nölling et al., 2001, it belongs to the saccharolytic group of genus *Clostridia* and can metabolize carbohydrates in order to produce a number of different commercially useful products especially acetone, butanol and ethanol. In the presence of oxygen in the area, *C. acetobutylicum* cannot grow since they are strictly anaerobic bacteria. However, vegetative cells may survive oxygen exposure for several hours (Gottschalk et al., 1981). The produced subterminal endospores also enabled it to survive in the environment for many years, even in the presence of oxygen. Although other members of the *Clostridia* produce some of the most lethal neurotoxins, *C. acetobutylicum* is a benign microorganism. Several reports have witnessed the production of acetone and butanol from this strain but however, none has identified justifiable adverse effects of the microorganism to the environment, neither to human and animal health. Therefore, in fermentation systems, this bacillus is used safely because of the low potential risk.

In this study, the most commonly studied solventogenic *Clostridia, Clostridium acetobutylicum* ATCC 824 was used as model bacteria. Prior to the isolation of this strain (from a Connecticut garden soil) in 1924, the Weizmann strain which had been in use in the early industrial production of acetone demonstrated close relationship with *C. acetobutylicum* (Nölling et al., 2001). Since this strain has been physiological characterized, it is used worldwide in a variety of molecular biology and metabolic engineering studies.

After reactivation of freeze-dried strain in a milk medium, it was cultivated on agar plates on a Lysogeny Broth (LB) medium in anaerobic conditions. The table below shows the components of LB medium.
Table 4. Components and their content for LB medium.

<table>
<thead>
<tr>
<th>Component</th>
<th>Formula</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td></td>
<td>1.50 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>Bacto tryptone</td>
<td></td>
<td>1.55 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>Glucose</td>
<td>C\textsubscript{6}H\textsubscript{12}O\textsubscript{6}</td>
<td>0.48 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>NaCl</td>
<td>0.39 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>Yeast extract</td>
<td></td>
<td>0.97 % (w/w\textsubscript{H2O})</td>
</tr>
</tbody>
</table>

The optimal temperature for cultivation of the strain is 37°C. A single colony obtained from an agar plates under anaerobic condition and cultivated in a liquid sterilized Clostridia Growth Medium (CGM) (Wiesenborn et al., 1988). The pH of CGM was adjusted to 5.0. The components of the liquid medium listed in Table 5.

Table 5. Components for Clostridia Growth Medium (CGM) (Wiesenborn et al., 1988).

<table>
<thead>
<tr>
<th>Component</th>
<th>Formula</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asparagine</td>
<td>C\textsubscript{4}H\textsubscript{8}N\textsubscript{2}O\textsubscript{3}</td>
<td>0.2 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>Potassium phosphate</td>
<td>K\textsubscript{2}HPO\textsubscript{4} · 3H\textsubscript{2}O</td>
<td>0.0982 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>NaCl</td>
<td>0.11 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}</td>
<td>0.2 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>Yeast extract</td>
<td></td>
<td>0.5 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>Glucose</td>
<td>C\textsubscript{6}H\textsubscript{12}O\textsubscript{6}</td>
<td>8.0 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>Monopotassium phosphate</td>
<td>KH\textsubscript{2}PO\textsubscript{4}</td>
<td>0.075 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>MnSO\textsubscript{4} · H\textsubscript{2}O</td>
<td>0.001 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>Iron sulphate</td>
<td>FeSO\textsubscript{4} · 7H\textsubscript{2}O</td>
<td>0.001 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>MgSO\textsubscript{4}</td>
<td>0.0348 % (w/w\textsubscript{H2O})</td>
</tr>
<tr>
<td>p-Aminobenzoic-acid</td>
<td>C\textsubscript{7}H\textsubscript{7}NO\textsubscript{2}</td>
<td>0.0004 % (w/w\textsubscript{H2O})</td>
</tr>
</tbody>
</table>

The Clostridia cells should have been ready for measurement after a couple of days at 37°C, were ready for measurements. However, because of unknown reason, the culture did not grow in the CGM media. Therefore, the measurements described herein were obtained from a culture which was grown in the liquid media whose components are displayed in Table 6.
Table 6. Liquid medium of *C. acetobutylicum* ATCC 824.

<table>
<thead>
<tr>
<th>Component</th>
<th>Formula</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monopotassium phosphate</td>
<td>KH$_2$PO$_4$</td>
<td>0.5 g/L</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>MgSO$_4$</td>
<td>0.2 g/L</td>
</tr>
<tr>
<td>(non-hydrous)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast extract</td>
<td></td>
<td>5 g/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>C$<em>6$H$</em>{12}$O$_6$</td>
<td>20-40 g/L</td>
</tr>
</tbody>
</table>

The culture is stored at room temperature and in a dark place until further use.

All the components of each media were weighed in precision scale and the liquid media were autoclaved under 100% N$_2$ gas for one hour at 120°C.
2. Preparation of biogas samples for Flow Cytometry and Electrooptical Measurements

In this study, the biogas samples were collected from the Institute for Agricultural and Urban Ecological Projects in Berlin (IASP). The regular feeding used in the reactor is done with maize silage (Agt bio energy GmbH) and some trace elements (MethaTrace, Biopract GmbH) are added.

The application of Flow Cytometry and Electrooptical Measurements for the analysis of biogas samples requires previous sample purification due to its high content of organic and inorganic particles and the presence of cell aggregates which could clog the capillary within the flow cytometer.

In order to prepare the samples for Flow Cytometry and Electrooptical measurements, the first procedure, which is used is easy and simple and includes only filtration steps (Biogas Preparation). The Biogas Preparation has three single steps. Firstly, the sample is diluted ten times with 0.09% NaCl. The second step is a filtration of the diluted sample using a filter with 160μm pore size and finally a vacuum filtration with 8μm filter is conducted with the filtrate. As shown in the Figure 12, there three possible samples which can be measured. These samples are stored at room temperature until further preparation for Flow Cytometry and Electrooptical measurements.

![Figure 12. Schematic figure illustrating the Biogas Preparation. Firstly, the biogas sample is diluted with NaCl. The first filtration is done with 160μm pore size filter and the filtrate is filtered under vacuum with 8μm filter. The three possible samples are ready for further Flow Cytometry preparation.](image)

Based on information from the literature, the second procedure for sample preparation is more complicated and contains also the fixation of samples (Nettmann et al., 2013). The purpose of fixation is to stabilize the cells near in life as possible as for reliable subsequent analysis. The fixed samples should be capable of...
withstanding further preparation steps and storage without changes in cell number, morphology or biochemical status. Furthermore, the fixation procedures should counteract the increase of autofluorescence, cell clogging, and distortion of surface characteristics.

The fixation starts with dilution of sample 1:10 with 0.09% NaCl. The diluted sample is filtered with 160μm pore size filter. Subsequently, according to Nettmann et al., 2013, 10mL of filtrate are fixed with 30mL of a 3.7% formaldehyde solution, which is diluted with Phosphate Buffered Saline (PBS) (pH=7.2). After 4 hours of storage at 4°C, the samples are centrifuged at 8,000×g for 20 minutes at room temperature. The supernatant is discarded and the pellet is washed twice in PBS (pH 7.2) using same centrifugation conditions as before. The pellet is re-suspended in 5mL PBS (pH 7.2) and is mixed with 5mL 96% ethanol. The fixed sample stored overnight at −20°C.

For 1L of solution of PBS is weighed 8.00g NaCl (M=58.44 g·L⁻¹), 1.44g Na₂HPO₄ (M=177.99 g·L⁻¹), 0.20g KCl (M=74.56 g·L⁻¹), and 0.24g KH₂PO₄ (M=136.09 g·L⁻¹). The pH is adjusted to 7.2 and the solution is filtered with 0.2μm pore size filter.

The optimized pre-treatment method for Flow Cytometry measurements includes the addition of a detergent, ultrasonic steps and filtration steps. The ordinary use of detergent is to accelerate the penetration of the fixatives into cells (Nettmann et al., 2013)

The optimized preparation starts with centrifugation of 10mL of fixed sample at 8,000×g for 20 min at room temperature. Using the same centrifugation conditions, the cell pellet is washed once with PBS (pH 7.2) to remove ethanol. The cell pellet is further re-suspended in 10mL 0.5% (w/v) sodium hexametaphosphate (SHMP) (pH 8.5). The sodium hexametaphosphate is filtered with 160μm pore size filter. After 10 min of incubation the sample is sonicated at 60 W for 1 min. The separation of microorganisms from organic or inorganic particles in the sample is conducted by a centrifugation step at 650×g for 2 min. After centrifugation, the supernatant contains free cells and is transferred to a sterile tube for further application. The cell pellet is re-suspended again in 10mL 0.5% (w/v) sodium hexametaphosphate (pH 8.5) and incubated for 10 min followed by a further ultrasonic treatment and centrifugation step. The incubation step, the ultrasound step, and the centrifugation step are repeated five times. After five repetitions, the remaining pellet should consist mainly of organic and inorganic material and a negligible quantity of free microbial cells. The free microbial cells are contained in supernatants in a sterile tube and are collected by centrifugation at 8,000×g for 20 min at room temperature. The supernatant is discarded and the pellet is re-suspended in 10mL PBS (pH 7.2). The next step is a vacuum filtration of the sample using 25μm pore size filter. The filter is washed with 40mL PBS (pH 7.2). The filtrate is centrifuged at 8,000×g for 20 min at room temperature. The supernatant is discarded and the pellet is re-
suspended in 10mL of PBS (pH 7.2) and is stored at room temperature until further preparation for Flow Cytometry and Electrooptical measurements. The measurements are carried out in the same day of preparation.

The diagram in Figure 13 depicts the steps of optimized preparation of fixed sample according Nettmann et al., 2013.

Figure 13. Schematic figure illustrating the design of optimized purification procedure. The fixed sample is centrifuged and is washed with PBS (pH=7.2). The pellet is re-suspended in SHMP solution. After incubation, the sample is sonicated and the supernatants are collected in a sterile tube. The pellet is re-suspended again in SHMP and incubated followed by a further ultrasonic treatment and centrifugation step. The incubation step, the ultrasound step, and the centrifugation step are repeated five times. The pooled supernatants are centrifuged to collect the free microbial cells. The pellet is re-suspended in PBS (pH=7.2) and a vacuum filtration with 25μm pore size filter is conducted. Finally, the filter is washed with PBS (pH=7.2) and filtrate is centrifuged. The pellet is ready for further use. (Source: Nettmann et al., 2013)

In this study, the above described procedure is investigated whether the addition of sodium hexametaphosphate and the ultrasound step is necessary for purification of biogas sample. Instead of 10mL 0.5% (w/v) sodium hexametaphosphate, 10mL of PBS (pH=7.2) is added instead. The ultrasonic step is replaced by vortex treatment for 30 sec. Thus, there were four different procedures to prepare the fixed samples. All applications were carried out in triplicates. Figures 14-18 show the procedures which were used to prepare the biogas samples for Flow Cytometry and Electrooptical measurements.
Figure 14. Flow diagram of Biogas Preparation of sample (Procedure 1).

Figure 15. Flow diagram of preparation of sample with sodium hexametaphosphate (SHMP) addition and ultrasonic treatment (Nettmann et al., 2013) (Procedure 2).

Figure 16. Flow diagram of preparation of sample with sodium hexametaphosphate (SHMP) addition and vortex treatment (Nettmann et al., 2013) (Procedure 3).
Before Flow Cytometry measurements all possible purified samples need to undergo an additional preparation because, as mentioned above, the bigger particles could clog the capillary within the flow cytometer. Thus, 2mL of purified sample is filtered under vacuum with a filter of 0.2μm pore size. The washing step of sample is done with 5mL PBS (pH=7.2) and the cells which are stuck on the filter are dissolved in a sterile tube with 10mL PBS. After vortex treatment, the OD is measured and adjusted to 0.1 with PBS (pH=7.2). The samples are ready now to be measured by Flow Cytometer.
The MACSQuantify™ Analyser (Miltenyi Biotec GmbH, Bergisch Gladbach, GER) was used to achieve all the flow cytometric analyses observed in this study. While the volume of the final sample was 200μL, the total volume of uptake sample which is soaked by the needle of cytometer was 45 μL. Regarding the other settings for the measurements, the maximum event rate was $10^4$ events per second and the analysis stopped after an absolute cell count of $10^6$ cells. The rate of flow was medium for all measurements without compensation. The calibration of MACSQuantify™ Analyser was done automatically with MACSQuant® Calibration Beads. The calibration solution consists of beads of 2-3μm size, which enable adjustment of the voltage settings of the flow cytometer. For scatter signals (FSC and SSC) a 488/10 bandpass filter was applied and the plots were displayed in log3 scale. For the detection of SYTO13 and BOX a 525/50 nm bandpass filter was applied while for the detection of PI, it was applied a 655-730 nm longpass filter. For the detection of DAPI a 450/50 nm bandpass filter was applied. The plots for all FL-signals were displayed in hlog scale.

The picture below depicts a representative MACSQuantify™ Analyser by Miltenyi Biotec GmbH, Bergisch Gladbach, GER.

![Picture 1. MACSQuantify™ Analyser (Miltenyi Biotec GmbH, Bergisch Gladbach, GER)]
3. Cell staining

In this study, for cell staining the dyes PI, DAPI, BOX and SYTO 13 are used. The first three dyes are provided by Sigma-Aldrich (Munich, GER) and SYTO 13 is purchased from Invitrogen. Table 7 shows all the conditions of preparation and storage of applied solutions.

Table 7. Conditions of preparation and storage of applied dyes. (*ddH₂O= double distilled water)

<table>
<thead>
<tr>
<th></th>
<th>PI</th>
<th>DAPI</th>
<th>BOX</th>
<th>SYTO 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of stock solution</td>
<td>1 mg/mL</td>
<td>10 mg/mL</td>
<td>5 mg/mL</td>
<td>5 mM</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>668.4 g/mol</td>
<td>350.25 g/mol</td>
<td>516.63 g/mol</td>
<td>~400 g/mol</td>
</tr>
<tr>
<td>Storage media, Temperature</td>
<td>ddH₂O, 4°C</td>
<td>ddH₂O, 4°C</td>
<td>Dimethyl Sulfoxide, -20°C</td>
<td>Dimethyl Sulfoxide, -20°C</td>
</tr>
<tr>
<td>Concentration of working solution</td>
<td>40 μg/mL</td>
<td>100 μg/mL</td>
<td>25 μg/mL</td>
<td>40 μM</td>
</tr>
</tbody>
</table>

All the solutions are stored in temperature indicated in the table above with appropriate media protected from light. The working solutions of dyes are prepared in the same day of measurements.

The staining optimized conditions are given in the following table.

Table 8. Staining conditions of fluorescent dyes. (*RT=Room Temperature)

<table>
<thead>
<tr>
<th></th>
<th>PI</th>
<th>DAPI</th>
<th>BOX</th>
<th>SYTO 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining time</td>
<td>2 min</td>
<td>30 min</td>
<td>4 min</td>
<td>2 min</td>
</tr>
<tr>
<td>Temperature</td>
<td>4°C</td>
<td>37°C</td>
<td>RT*</td>
<td>RT*</td>
</tr>
<tr>
<td>Final concentration of fluorescent dye</td>
<td>1 μg/mL</td>
<td>20 μg/mL</td>
<td>1 μM</td>
<td>3 μM</td>
</tr>
</tbody>
</table>

All Flow Cytometry measurements were performed in 1.5mL Eppendorf tubes and are carried out in triplicates. As mentioned above the volume of final sample was 200μL for unstained and stained samples.

In Table 9 represents the volumes of stained and unstained samples used for Flow Cytometry measurements and cell staining.
Table 9. Volumes of stained and unstained samples used for cell staining

<table>
<thead>
<tr>
<th>Dye (μL)</th>
<th>Culture (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>unstained</td>
<td>200</td>
</tr>
<tr>
<td>PI</td>
<td>5</td>
</tr>
<tr>
<td>DAPI</td>
<td>40</td>
</tr>
<tr>
<td>BOX</td>
<td>4</td>
</tr>
<tr>
<td>SYTO 13</td>
<td>15</td>
</tr>
</tbody>
</table>
4. Preparation of positive and negative controls for biogas and bacteria samples

The preparation of positive and negative controls is similar for both biogas and bacteria samples. For the negative control, the unstained biogas and bacteria sample respectively is used.

For the positive control, various methods are used. The first control is the heat shock of samples for 30 minutes at 99°C. The addition of butanol to a final concentration of 200mM to the sample is also used as a method. The last method, which is used only with bacteria samples, is the addition of aceton to a final concentration of 100mM. For heat shock a heating block Thermomixer comfort (Eppendorf AG, Hamburg, GER) is used. All methods for positive control are aimed at loss of cell viability and are stained with all mentioned dyes.
5. Electrooptical measurements of biogas samples (EloTrace)

As mentioned in Theoretical background, using EloTrace it is measured the polarizability, which is closely linked to the actual physiological state of the cell.

Before Electrooptical measurements all possible purified samples don’t need to undergo a specific difficult preparation. The only needed step is to dilute the samples with 0.09% NaCl to adjust the OD=0.1. About the device, it is also necessary to check the installation and filter set and washing step. All optical detectors of EloTrace (ODFerm, ODInput, ODOoutput) and the electro-optical measuring unit must be recalibrated after the system restarts.

Each measurement cycle includes repetitive procedures, which are the cleaning of lines and vessels, the sampling, the sample preparation, the electrooptical measurement, the cleaning and finally the waiting until to the next cycle. The minimum duration for all these procedures for EloTrace 3.0 is about 12-15 minutes. After the end of measurements the fluidic system of EloTrace 3.0 has to be cleaned.

The picture of the EloTrace 3.0 sampling instrument used in this study is shown below.

![Picture 2. EloTrace 3.0 (TU Berlin). In the right there is a pump for receiving samples. The filtration unit is shown in the middle of the picture. In the left side it is shown three pumps for sample dilution and forwarding into the measuring cell.]
Results

1. Optimization of the sample preparation procedure for Flow Cytometry

While the staining conditions of the three dyes PI, BOX and SYTO 13 for biogas samples were based on protocol for *Corynebacterium glutamicum* cultivations, the staining conditions of DAPI needed to be found. As mentioned in Theoretical background, DAPI is a popular nucleic acid stain used in Flow Cytometry. It can be used to stain both live and fixed cells, but it passes through the membrane less efficiently in live cells. In Table 10 is summarized the optimal staining temperature, the final concentration of fluorescent dye and the staining time for staining of biogas samples for the three dyes PI, BOX and SYTO 13 (Protocol for *Corynebacterium glutamicum* cultivations).

Table 10. Optimal staining conditions of fluorescent dyes, PI, BOX and DAPI. (*RT=Room Temperature)

<table>
<thead>
<tr>
<th>Dye</th>
<th>Temperature</th>
<th>Final concentration</th>
<th>Staining time</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td>4°C</td>
<td>1 μg/mL</td>
<td>2 min</td>
</tr>
<tr>
<td>BOX</td>
<td>RT*</td>
<td>1 μM</td>
<td>4 min</td>
</tr>
<tr>
<td>SYTO 13</td>
<td>RT*</td>
<td>3 μM</td>
<td>2 min</td>
</tr>
</tbody>
</table>

The available literature of the sample preparation procedure for Flow Cytometry and especially the conditions of DAPI staining in bacterial cells are provided in the following Table.

Table 11. Sample preparation staining procedures of DAPI staining of bacteria cells. (*RT=Room Temperature)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Concentration</th>
<th>Staining time</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT* in dark</td>
<td>10 μg/mL</td>
<td>40 min</td>
<td>(Yu, et al; 1995)</td>
</tr>
<tr>
<td>RT* in dark</td>
<td>5 μg/mL</td>
<td>15 min</td>
<td>(Epstein and Rossel; 1995)</td>
</tr>
<tr>
<td>RT* in dark</td>
<td>1 μg/mL</td>
<td>5 min</td>
<td>(Hoff; 1988)</td>
</tr>
<tr>
<td>RT* in dark</td>
<td>1 μg/mL</td>
<td>10 min</td>
<td>(Pozarowski and Darzynkiewicz; 2004)</td>
</tr>
<tr>
<td>RT* in dark</td>
<td>0.5 μg/mL</td>
<td>10 min</td>
<td>(Saby, et al; 1997)</td>
</tr>
</tbody>
</table>
As shown in Table 11, there is not a defined sample preparation and consequently it is necessary to determine the variables and their sensitivity to the methods so as the optimal conditions for DAPI staining are defined. The variables are the staining temperature, the final concentration of fluorescent dye and the staining time. The response is the percentage of staining cells. The staining temperature is considered a main variable of performance of the staining, because of the effect in metabolism of bacterial cells. The optimization of the staining time is essential to be determined the potential toxicity of the dye and the needed time of interaction and reaction of dye to cells. The third variable, the final concentration of dye can readily be the limiting factor of response and in this case of the amount of staining cells. Therefore the concentration of dye has to be such as not to implicate toxic effects as is the loss of cell viability, or a non-specific binding which is the shifting of the unstained population.

The determination of the variables and their sensitivity for the DAPI staining optimization was performed by Design of Experiments (DoE). The experiment is defined with three factors and two levels, one low and one high, so a design of 3² factorial (8 experiences). The reproducibility of the experiment is done by defining a center point and thus resulting eleven (11) experiments. Using DoE, taken as the maximum amount of information making as few experiments. The experiments are made in a completely random sequence and in triplicates for statistical purposes. Apart from these eleven experiments for optimization of DAPI staining, cells without DAPI are measured at several time points in order to notice, if various cell characteristics are changed due to temperature, storage time or other factors.

For the Design of Experiments, the program MODDE v. 10.0, MKS Umetrics AB, is used. This program provides different tools such as Summary of Fit, Contour plot and Coefficient plot, for data analysis and its interpretation.

Using Summary of Fit, the summary statistics are presented in four parameters which are R², Q², Model validity, and Reproducibility. The first two columns are R² and Q² and should be close in size. In most situations, the difference shouldn't be more than 20%. All parameters values are between 0 and 1, which is the perfect one.

The first column is R² and shows the model fit. It is considered the fraction of the variation of the response explained by the model. R² overestimates the goodness of fit. For example, a model with R² of 0.5 is a model with rather low significance.

The second column is Q² and shows an estimate of the future prediction precision. It is considered the fraction of the variation of the response predicted by the model according to cross validation. As opposed to the R², Q² underestimates the goodness of fit and can be negative for very poor models. Q² should be greater than
0.1 for a significant model and greater than 0.5 for a good model. It is assessed the best and most sensitive indicator. When R2 and Q2 values are 1, the model is very good with excellent predictive power.

The third column is Model validity and is a test of diverse model problems. A value less than 0.25 for Model validity indicates statistically significant model problems or that a term is missing. When the pure error is very small, the Model validity can be low even though the model is good and complete. When the Model validity value is larger than 0.25, there is no lack of fit of the model and the model error is in the same range as the pure error. For example, the Model validity might be low in very good models when they have Q2>0.9, due to high sensitivity in the test or extremely good replicates. This factor also presents a perfect model when its value is 1.

The final column is Reproducibility and is the variation of the replicates compared to overall variability. The Reproducibility is calculated at the center points comparing to the total variation of the response. The value should be greater than 0.5 and the perfect one is 1.

The optimization of DAPI staining was done in two experimental design steps using Clostridium acetobutylicum cells. The first step is the performance of an experimental design. In the Table 12 is shown the design matrix for the optimization.

---

Table 12. First design matrix for optimization of DAPI staining.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>5</td>
</tr>
<tr>
<td>Final concentration (μg/mL)</td>
<td>20</td>
</tr>
<tr>
<td>Staining time (min)</td>
<td>2</td>
</tr>
</tbody>
</table>

According to the literature, the temperature of staining is 22°C, namely room temperature. In the experimental design, a temperature range from 5°C, namely on ice, where the cells stay in a dormant state, until 37°C has been chosen. Despite the fact that the final concentration of dye vary between the experiments, a broader range with values considerably higher than those mentioned in the literature has been selected. Finally, regarding to the staining time, it has been considered that the appropriate range for the first experimental design was from 2 minutes to 20 minutes.
The Table below shows the eleven experiments of the first DoE experimental design approach for DAPI staining. As shown, the experiments were performed in random order.

**Table 13. Experiments of the first DoE experimental design approach for DAPI staining.**

<table>
<thead>
<tr>
<th>Exp. No</th>
<th>Exp. Name</th>
<th>Run Order</th>
<th>Temperature (°C)</th>
<th>Final concentration (μg/mL)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N1</td>
<td>9</td>
<td>5</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>N2</td>
<td>8</td>
<td>5</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>N3</td>
<td>7</td>
<td>37</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>N4</td>
<td>1</td>
<td>37</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>N5</td>
<td>3</td>
<td>5</td>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>N6</td>
<td>11</td>
<td>5</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>N7</td>
<td>10</td>
<td>37</td>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>N8</td>
<td>2</td>
<td>37</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>N9</td>
<td>5</td>
<td>24</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>N10</td>
<td>4</td>
<td>24</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>N11</td>
<td>6</td>
<td>24</td>
<td>40</td>
<td>10</td>
</tr>
</tbody>
</table>

During these experiments and at several time points, measurements for positive and negative control were performed. As mentioned in Material and Methods section, the negative control is the measurement of unstained cells. About positive control for these experiments, all three methods, heat shock, butanol and aceton, are made at random sequence. The response of DAPI staining experiments namely the percentage of staining cells is presented in the following Table.
Table 14. Results of DAPI staining experiments of the first DoE experimental design approach. % DAPI (+) is the percentage of staining cells using the DAPI dye.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% DAPI (+)</th>
<th>Heat shock % DAPI (+)</th>
<th>Aceton % DAPI (+)</th>
<th>Butanol % DAPI (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0.01</td>
<td>0.09</td>
<td>0.64</td>
<td>2.52</td>
</tr>
<tr>
<td>N1</td>
<td>22.91</td>
<td>34.24</td>
<td>20.17</td>
<td>26.88</td>
</tr>
<tr>
<td>N2</td>
<td>24.16</td>
<td>38.83</td>
<td>23.77</td>
<td>28.93</td>
</tr>
<tr>
<td>N3</td>
<td>15.89</td>
<td>39.30</td>
<td>20.80</td>
<td>28.83</td>
</tr>
<tr>
<td>N4</td>
<td>28.57</td>
<td>43.04</td>
<td>29.00</td>
<td>28.96</td>
</tr>
<tr>
<td>N5</td>
<td>21.45</td>
<td>22.83</td>
<td>19.42</td>
<td>23.04</td>
</tr>
<tr>
<td>N6</td>
<td>19.07</td>
<td>21.22</td>
<td>23.18</td>
<td>18.00</td>
</tr>
<tr>
<td>N7</td>
<td>17.81</td>
<td>26.45</td>
<td>18.51</td>
<td>19.84</td>
</tr>
<tr>
<td>N8</td>
<td>19.77</td>
<td>27.00</td>
<td>22.01</td>
<td>19.81</td>
</tr>
<tr>
<td>N9</td>
<td>22.22</td>
<td>32.12</td>
<td>22.58</td>
<td>21.36</td>
</tr>
<tr>
<td>N10</td>
<td>22.89</td>
<td>31.99</td>
<td>23.49</td>
<td>24.67</td>
</tr>
<tr>
<td>N11</td>
<td>24.01</td>
<td>29.58</td>
<td>22.65</td>
<td>25.94</td>
</tr>
</tbody>
</table>

Regarding to positive control, the heat shock has better results than the other two methods and was considered the appropriate positive control for experiments. The addition of butanol has also good results, but not so high values as heat shock.

The first plot, the Replicates plot, shows the variation in results for all experiments in order to provide a quick overview of raw data. The values of the responses (green and blue points) are plotted vs. experimental runs displaying the variation in the response for replicated experiments. The ideal outcome is that the variability of the repeated experiments is much less than the overall variability. In Figure 19, it is observed that the percentages of blue points which are the replicated experiments are close enough and their variability is much less than the overall variability.
The significance of the model terms has to be analyzed. The Coefficient plot presents a graphical representation of the model terms in order to determine their significance. The Coefficient Plot displays the coefficients for the selected response with the confidence interval as error bars. The coefficients refer to the data, which are scaled and centered. Particularly, a significant term is one with a large distance from y=0, in this case that means a high percentage, either positive or negative, as well as having an uncertainty level that does not extend across y=0. Instead, a non significant model term is a model term close to y=0 and with an uncertainty level that crosses y=0. Figure 20 shows the comparison of coefficients of the first DoE approach.

(*Tim=Staining time, Temp=Staining Temperature, Con=Final Concentration of dye)
As shown, the staining temperature has almost no influence on the results. Although the confidence interval of the staining time and the final concentration of DAPI includes zero, these two terms of model are significant because their percentages are higher than the percentage of staining temperature.

Other used plot is the Observed vs. Predicted Plot, which displays obviously the observed values vs. the predicted values. The model is good, if the points of the plot are close to a straight line. Additionally, if the Degrees of Freedom is under 3 (DF < 3), the plot will implicitly give a perfect fit. In the Figure below, the points are close enough in the straight line and the model is considered good.

![Observed vs. Predicted Plot for DAPI staining of the first DoE approach.](image)

The 4D Contour plot displays the predicted response values for the selected response, spanned by two factors, in 9 response surface contour plots in a 3x3 grid and spanned by another two factors. Figure 22 depicts the response values as derived from DoE approach.
Finally, the validity of the regression model is observed by the Summary of Fit plot, where the R2, Q2, Model validity and Reproducibility are displayed (Figure 23).

The experimental data is well described by the regression model. It is obtained a high R2 and a good Reproducibility, close to 1. The Model validity is above 0.25, but not perfect. The negative Q2 means that there are some non-necessary terms, which have poor sensitivity, in the model. Especially, the staining temperature was considered to be non-sensitive for the model and it can be removed.
The outcome of the first DoE plan is that the staining temperature is a non-significant parameter. The need to find a narrow range for the other two variables led to the design of a second DoE approach. In this experimental design, the temperature was constant at 37°C, because the fourth experiment (37°C, 20 μg/mL and 20 min, Table 13) had the best percentages about staining cells as it shown in bold in Table 14. The results for Flow Cytometry measurements are shown in Figure 24 for unstained cells and in Figure 25 for DAPI staining.

Figure 24. Flow Cytometry measurements for unstained Clostridium acetobutylicum cells. The left diagrams show the sample measurement and the three possible positive controls, while on the right shows the relevant histograms.
Figure 25. Flow Cytometry measurements for N4 experiment (37°C, 20 μg/mL and 20 min). The left diagrams show the sample measurement and the three possible positive controls with DAPI staining while on the right shows the relevant histograms.

The design matrix of the second DoE approach for the optimization of DAPI staining is shown in the Table below.

Table 15. Second design matrix for optimization of DAPI staining.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final concentration (μg/mL)</td>
<td>1 20</td>
</tr>
<tr>
<td>Staining time (min)</td>
<td>2 30</td>
</tr>
</tbody>
</table>

In this plan, the values of final concentration of dye were lower than the values in the first DoE, because it seemed that 20 μg/mL was the appropriate value of concentration and it is needed to be checked the results with lower values of concentration. Regarding the staining time, in the second approach a broader range has been selected with the greater value the 30 minutes. It is considered that a greater staining time value than 30 minutes is not practical.

Table 16 shows the seven experiments of the second DoE experimental design approach for DAPI staining. The experiments were performed in random order.
**Table 16. Experiments of the second DoE experimental design approach for DAPI staining.**

<table>
<thead>
<tr>
<th>Exp. No</th>
<th>Exp. Name</th>
<th>Run Order</th>
<th>Temperature (°C)</th>
<th>Final concentration (μg/mL)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N1</td>
<td>3</td>
<td>37</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>N2</td>
<td>5</td>
<td>37</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>N3</td>
<td>4</td>
<td>37</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>N4</td>
<td>2</td>
<td>37</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>N5</td>
<td>7</td>
<td>37</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>N6</td>
<td>1</td>
<td>37</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>N7</td>
<td>6</td>
<td>37</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

The percentage of staining cells is presented in Table 17. In these experiments the only positive control method used was the heat shock.

**Table 17. Results of DAPI staining experiments of the second DoE experimental design approach. % DAPI (+) is the percentage of staining cells using the DAPI dye.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>% DAPI (+)</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Heat shock</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% DAPI (+)</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>N1</td>
<td>21.39</td>
<td>22.36</td>
</tr>
<tr>
<td>N2</td>
<td>26.67</td>
<td>22.85</td>
</tr>
<tr>
<td>N3</td>
<td>36.59</td>
<td>41.24</td>
</tr>
<tr>
<td><strong>N4</strong></td>
<td><strong>38.60</strong></td>
<td><strong>48.02</strong></td>
</tr>
<tr>
<td>N5</td>
<td>40.03</td>
<td>39.66</td>
</tr>
<tr>
<td>N6</td>
<td>38.96</td>
<td>39.52</td>
</tr>
<tr>
<td>N7</td>
<td>39.89</td>
<td>38.89</td>
</tr>
</tbody>
</table>

Firstly, a quick overview of raw data is shown in Figure 26. The values of the responses (green and blue points) are plotted vs. experimental runs displaying the variation in the response for replicated experiments. The ideal outcome is that the variability of the repeated experiments is much less than the overall variability. As in the first DoE plan, the percentages of the replicated experiments are almost the same and therefore the outcome is ideal.
Figure 26. Quick overview of the raw data of second DoE.

Figure 27 shows the comparison of coefficients of the second DoE approach.

Figure 27. Scaled and centered Coefficients for DAPI staining of the second DoE approach. (*Tim=Staining time, Con=Final Concentration of dye)

In Figure 27, the Coefficients plot is consistent with the results obtained in the first DoE plan. The concentration of dye has higher sensitivity for the model than the staining time.
The Observed vs. Predicted Plot for second DoE plan is shown in Figure 28. The Degrees of Freedom is three and consequently the plot will implicitly give a perfect fit.

Figure 28. Observed vs. Predicted Plot for DAPI staining of the second DoE approach.

Moreover, in order to find out about the dependency between the DAPI concentration and the staining time, a contour plot has been used. The contour plot of these two factors shows that the percentage of stained cells increases with the concentration of dye and the optimal staining time is more than 20 minutes.

Figure 29. Contour plot of response values for DAPI staining of the second DoE approach. (*Con=Final Concentration of dye)
The Summary of Fit of the model is displayed in Figure 30 and especially the interaction between the staining concentration and the temperature is shown.

As shown, the experimental data is well described by the regression model. R2 is above 0.5 and a good Reproducibility, really close to 1, is obtained. R2 and Q2 are both low and so the predictability of the model is rather poor. The purpose of DoE plan was to find out the best conditions of DAPI staining and not to obtain a model for simulation. Although the final results are poor, they reflect good staining conditions.

Taking into account the percentages of staining cells from second DoE design in Table 17, the optimal final concentration of dye is 20 μg/mL and the optimal staining time is 30 minutes (Tables 16). The results for Flow Cytometry measurements are shown in Figure 31 for unstained cells and DAPI staining.
Figure 31. Flow Cytometry measurements for optimal conditions for DAPI staining for *Clostridium acetobutylicum* cells. The first four diagrams are referred to unstained sample and as positive control is used the heat shock whereas the right diagrams show the influence of DAPI staining to the cells.

Therefore, the conclusion of two experimental designs is that the optimal conditions for DAPI staining are 37°C as staining temperature, 20 μg/mL as final concentration of DAPI and 30 minutes as staining time.
2. DAPI staining

As mentioned in a previous chapter, *Clostridium acetobutylicum* is one of some *Clostridium* strains - saccharolytic butyric acid-producing bacteria and is able to metabolize a great variety of substrates, pentoses, hexoses, mono-, di- and polysaccharides (Jones and Woods, 1986).

The strictly anaerobic *Clostridium acetobutylicum* is a promising biofuel producer due to its capacity to ferment a variety of carbohydrates into acetone, butanol, and ethanol which are important chemicals used in a plenty of industrial applications. The metabolism of this organism is characterized by two distinct and sequential phases.

The first phase, the acidogenic phase, occurs during exponential growth and involves the rapid production of cells, hydrogen, carbon dioxide, acetic acid and butyric acid accompanied by a decrease in culture pH value. During the acidogenesis, the acids production ensures high ATP and NADH yields. The reduction in pH and therefore the increase in concentrations of acids helps the metabolism of cells to shift to the second phase and the solvent production known as the solventogenic phase.

In the solventogenic phase, the culture produces butanol, acetone, and ethanol as the culture enters into the stationary phase. Butyrate and acetate are typically re-assimilated to produce solvents, thus raising the pH of the culture. During the solventogenesis the active cells become endospores unable to reproduce themselves. This shift towards solvent production is thought to be a way for the culture to detoxify the culture environment of the harmful acids towards the less damaging solvents. This metabolic phase transition is also accompanied by changes in transcriptome, proteome, and metabolome. In the both batch and continuous cultures of the wildtype strain, peak solvent production occurs at low pH, between 4.5 and 5.0, but as extracellular pH is increased solvent production decreases and production of acetate and butyrate is elevated. The understanding of this pH dependence is still not fully understood.

The FSC-SSC and FSC-VioBlue plots for *Clostridium acetobutylicum* are shown below as taken from Flow Cytometry measurements. In these diagrams, the two distinct phases of bacteria are clearly shown.
Figure 32. FSC-SSC and FSC-VioBlue plots for *Clostridium acetobutylicum*. In the left diagram, the left circle depicts the acidogenic phase and the right one the solventogenic phase. In the right diagram, the cells which are in the left side of vertical line belong to acidogenic phase while the shift of cells is the solventogenic phase.

The SSC-FSC plot of biogas samples is the same for all procedures of preparation of the samples for Flow Cytometry and Electrooptical measurements (Figure 33).

Figure 33. SSC-FSC plot for biogas samples.

As mentioned in the Material and Methods section, there are five different procedures used in this study. The results for DAPI staining from Flow Cytometry measurements are presented below for all preparations.
Procedure 1 - Biogas Preparation of sample

In this procedure, the possible samples which can be measured are three and they are the 160μm filtrate sample, the 8μm filter cake sample and the 8μm filtrate sample. Figures 34-36 depict the results of Flow Cytometry measurements for unstained cells and DAPI staining.

160μm filtrate sample

Figure 34. Flow Cytometry measurements for 160μm filtrate sample. The first six diagrams are referred to unstained sample and as positive control is used the heat shock and the butanol addition whereas the right diagrams show the influence of DAPI staining to the same cells.

8μm filter cake sample

Figure 35. Flow Cytometry measurements for 8μm filter cake sample. The first six diagrams are referred to unstained sample and as positive control is used the heat shock and the butanol addition whereas the right diagrams show the influence of DAPI staining to the same cells.
**8μm filtrate sample**

![Flow Cytometry measurements for 8μm filtrate sample.](image)

According to these plots, it is noticed that the 8μm filtrate sample has less amount of cells than the other two samples and therefore the results are not reliable. On the other hand, the 160μm filtrate sample and the 8μm filter cake sample have similar results. The methods used as positive control are both successful because the clear shift of culture in both samples helps to compare the staining sample.

The other four procedures are based to the optimized preparation of fixed sample according Nettmann et al., 2013. As mentioned above, the two parameters, which are changed to ascertain, whether they are necessary for purification of biogas samples, are the addition of the detergent and the ultrasonic step. Figures 37-40 depict the results of Flow Cytometry measurements for unstained cells and DAPI staining for these four procedures.

**Procedure 2 - Preparation of sample with sodium hexametaphosphate (SHMP) addition and ultrasonic treatment (Nettmann et al., 2013)**

In Figure 37, it is shown the results of fixed sample with DAPI staining compared with unstained biogas cells. The preparation includes the addition of detergent and it is used the ultrasonic treatment. The heat shock and the addition of butanol are both effective for this sample.
Figure 37. Flow Cytometry measurements for preparation of sample with sodium hexametaphosphate (SHMP) addition and ultrasonic treatment (Nettmann et al., 2013). The first six diagrams are referred to unstained sample and as positive control is used the heat shock and the butanol addition whereas the right diagrams show the influence of DAPI staining to the same cells.

Procedure 3 - Preparation of sample with sodium hexametaphosphate (SHMP) addition and vortex treatment (Nettmann et al., 2013)

In Figure 38, it is shown the results of fixed sample with DAPI staining compared with unstained biogas cells. The preparation includes the addition of detergent and it is used the vortex step. The heat shock and the addition of butanol are both effective for this sample.

Figure 38. Flow Cytometry measurements for preparation of sample with sodium hexametaphosphate (SHMP) addition and vortex treatment (Nettmann et al., 2013). The first six diagrams are referred to unstained sample and as positive control is used the heat shock and the butanol addition whereas the right diagrams show the influence of DAPI staining to the same cells.
Procedure 4 - Preparation of sample with PBS (pH=7.2) addition and ultrasonic treatment (Nettmann et al., 2013)

In Figure 39, it is shown the results of fixed sample with DAPI staining compared with unstained biogas cells. The preparation includes the addition of PBS (pH=7.2) instead of detergent and it is used the ultrasonic step. The heat shock and the addition of butanol are both effective for this sample.

Figure 39. Flow Cytometry measurements for preparation of sample with PBS (pH=7.2) addition and ultrasonic treatment (Nettmann et al., 2013). The first six diagrams are referred to unstained sample and as positive control is used the heat shock and the butanol addition whereas the right diagrams show the influence of DAPI staining to the same cells.

Procedure 5 - Preparation of sample with PBS (pH=7.2) addition and vortex treatment (Nettmann et al., 2013)

In Figure 40, it is shown the results of fixed sample with DAPI staining compared with unstained biogas cells. The preparation includes the addition of PBS (pH=7.2) instead of detergent and it is used the vortex treatment. The heat shock and the addition of butanol are both effective for this sample.
Flow Cytometry measurements for preparation of sample with PBS (pH=7.2) addition and vortex treatment (Nettmann et al., 2013). The first six diagrams are referred to unstained sample and as positive control is used the heat shock and the butanol addition whereas the right diagrams show the influence of DAPI staining to the same cells.

In the four last procedures, it is noticed that the results with sodium hexametaphosphate (SHMP) compared to results with PBS (pH=7.2) instead of detergent are similar. The percentages of DAPI staining are approximately 17% in all procedures. Thus, the conclusion is that the addition of detergent has no influence to the results. Moreover, there is no difference in the results with ultrasonic treatment and the results with vortex step. Likewise, the conclusion of these results is that the ultrasonic and vortex treatment has no influence for the results. In these procedures an absolute cell count of $10^6$ cells are measured. The heat shock and the addition of butanol used as positive control are both successful because the clear shift of culture in all samples helps to compare the staining sample.
3. BOX staining

The analysis of five different procedures used in this study is done also using BOX staining. As opposed to DAPI, which stain all DNA, both live and fixed cells, BOX is used to check the vitality of cells because when cells lose their membrane potential total or partially, BOX can passively diffuse through the cell membrane and binds to positively charged proteins or unspecifically to hydrophobic regions. It cannot bind to the cell wall or outer membrane structures of living cells. The results for BOX staining from Flow Cytometry measurements are presented below for all preparations.

Procedure 1 - Biogas Preparation of sample

In this procedure, the possible samples which can be measured are three and they are the 160μm filtrate sample, the 8μm filter cake sample and the 8μm filtrate sample. Figures 41-43 depict the results of Flow Cytometry measurements for unstained cells and BOX staining.

160μm filtrate sample

![Figure 41. Flow Cytometry measurements for 160μm filtrate sample. The first six diagrams are referred to unstained sample and as positive control is used the heat shock and the butanol addition whereas the right diagrams show the influence of BOX staining to the same cells.](image-url)
Figure 42. Flow Cytometry measurements for 8μm filter cake sample. The first six diagrams are referred to unstained sample and as positive control is used the heat shock and the butanol addition whereas the right diagrams show the influence of BOX staining to the same cells.

Figure 43. Flow Cytometry measurements for 8μm filtrate sample. The first six diagrams are referred to unstained sample and as positive control is used the heat shock and the butanol addition whereas the right diagrams show the influence of BOX staining to the same cells.

According to these plots, it is also noticed that the 8μm filtrate sample has less amount of cells than the other two samples and therefore the results are not so reliable. On the other hand, the 160μm filtrate sample and the 8μm filter cake sample have similar results. The percentages of BOX staining are approximately 82% in both procedures. The heat shock and the addition of butanol used as positive control are both successful because the clear shift of culture in both samples helps
to compare the staining sample. In this case, the shift of culture is clearer than the results of DAPI staining.

Figures 44-47 depict the results of Flow Cytometry measurements for unstained cells and BOX staining for the other four procedures based to the optimized preparation of fixed sample according Nettmann et al., 2013.

Procedure 2 - Preparation of sample with sodium hexametaphosphate (SHMP) addition and ultrasonic treatment (Nettmann et al., 2013)

In Figure 44, it is shown the results of fixed sample with BOX staining compared with unstained biogas cells. The preparation includes the addition of detergent and it is used the ultrasonic treatment. The heat shock and the addition of butanol are both effective for this sample.

![Figure 44. Flow Cytometry measurements for preparation of sample with sodium hexametaphosphate (SHMP) addition and ultrasonic treatment (Nettmann et al., 2013). The first six diagrams are refered to unstained sample and as positive control is used the heat shock and the butanol addition whereas the right diagrams show the influence of BOX staining to the same cells.](image)

Procedure 3 - Preparation of sample with sodium hexametaphosphate (SHMP) addition and vortex treatment (Nettmann et al., 2013)

In Figure 45, it is shown the results of fixed sample with BOX staining compared with unstained biogas cells. The preparation includes the addition of detergent and it is used the vortex step. The heat shock and the addition of butanol are both effective for this sample.
Figure 45. Flow Cytometry measurements for preparation of sample with sodium hexametaphosphate (SHMP) addition and vortex treatment (Nettmann et al., 2013). The first six diagrams are referred to unstained sample and as positive control is used the heat shock and the butanol addition whereas the right diagrams show the influence of BOX staining to the same cells.

Procedure 4 - Preparation of sample with PBS (pH=7.2) addition and ultrasonic treatment (Nettmann et al., 2013)

In Figure 46, it is shown the results of fixed sample with BOX staining compared with unstained biogas cells. The preparation includes the addition of PBS (pH=7.2) instead of detergent and it is used the ultrasonic step. The heat shock and the addition of butanol are both effective for this sample.

Figure 46. Flow Cytometry measurements for preparation of sample with PBS (pH=7.2) addition and ultrasonic treatment (Nettmann et al., 2013). The first six diagrams are referred to unstained sample and as positive control is used the heat shock and the butanol addition whereas the right diagrams show the influence of BOX staining to the same cells.
Procedure 5 - Preparation of sample with PBS (pH=7.2) addition and vortex treatment (Nettmann et al., 2013)

In Figure 47, it is shown the results of fixed sample with BOX staining compared with unstained biogas cells. The preparation includes the addition of PBS (pH=7.2) instead of detergent and it is used the vortex treatment. The heat shock and the addition of butanol are both effective for this sample.

Figure 47. Flow Cytometry measurements for preparation of sample with PBS (pH=7.2) addition and vortex treatment (Nettmann et al., 2013). The first six diagrams are referred to unstained sample and as positive control is used the heat shock and the butanol addition whereas the right diagrams show the influence of BOX staining to the same cells.

In the four last procedures, it is noticed that the results with sodium hexametaphosphate (SHMP) compared to results with PBS (pH=7.2) instead of detergent are also similar like the results of DAPI staining. Thus, the conclusion is the same, namely that the addition of detergent has no influence to the results. Moreover, the results with ultrasonic treatment and the results with vortex treatment are also similar. Likewise, the conclusion of these results is that the ultrasonic and vortex treatment has no influence to the results. The percentages of BOX staining are approximately 87% in all procedures. The heat shock and the addition of butanol used as positive control are both successful because the clear shift of culture in all samples helps to compare the staining sample.
4. PI and SYTO 13 staining

In the Material and Methods chapter, other two fluorescent dyes are mentioned. They were used with all five procedures, but the results were not satisfactory and useful for the study. These dyes are PI and SYTO 13.

As mentioned, PI is used to evaluate cell viability or DNA content in cell cycle analysis. It can enter cells, which have lost their membrane integrity and stain the DNA. Therefore, PI is used to evaluate the cellular viability. The results of Flow Cytometry measurements for all procedures are same and are depicted in Figure below.

![Unstained sample vs PI staining](image)

**Figure 48.** SSC-PI plots for unstained biogas sample and PI staining biogas sample. The left diagram shows the unstained culture in the PI channel whereas the right diagram shows the influence of PI staining in the biogas sample.

It is obvious that this dye has no influence in biogas samples. The reasons of this behavior are not clear because PI stains the dead cells of culture. It is not possible all of the cells are alive and it requires further research.

SYTO 13 can be used to stain DNA and RNA in both live and dead eukaryotic cells, in gram-positive and gram-negative bacteria. It is not useful in this study because it is needed to know the cellular viability of samples and therefore the results of BOX staining are used. In Figure 49 it is shown the SYTO staining after Flow Cytometry measurement.
Figure 49. SSC-FITC plots for unstained biogas sample and SYTO 13 staining biogas sample. The left diagram shows the unstained culture in the SYTO channel whereas the right diagram shows the influence of SYTO 13 staining in the biogas sample.

Looking at these plots the shift of stained cells is clear as opposed to the diagrams with PI staining. Moreover, the results of SYTO 13 staining are quite similar in all preparations of biogas samples.
5. Comparison of Flow Cytometry and Electrooptical measurements

In parallel are carried out Electrooptical measurements using EloTrace 3.0. As mentioned, EloTrace is the first world-wide automatic electrooptical measurement system for the determination of cell activity, stress levels, cell size and morphological of bacterial cells. The level of the polarizability of cells is closed linked to the actual physiological state of the cell. The results from these measurements are treated and presented in this chapter.

Firstly, the results from Biogas Preparation of biogas sample are presented. Figures 50-52 depict the electrooptical signal of three possible sample of this procedure.

Procedure 1 - Biogas Preparation of sample

The relation of signal strength and frequency of electrical field for 160µm filtrate sample is shown in Figure 50.

160µm filtrate sample

![Electrooptical Signal for 160µm filtrate sample](image)

**Figure 50.** Electrooptical signal for 160µm filtrate sample. It is shown the relation of signal strength and frequency of electrical field.

8µm filter cake sample

The relation of signal strength and frequency of electrical field for 8µm filter cake sample is shown in Figure 51.
Figure 51. Electrooptical signal for 8μm filter cake sample. It is shown the relation of signal strength and frequency of electrical field.

8μm filtrate sample

The relation of signal strength and frequency of electrical field for 8μm filtrate sample is shown in Figure 52.

Figure 52. Electrooptical signal for 8μm filtrate sample. It is shown the relation of signal strength and frequency of electrical field.
The 8µm filtrate results cannot be used, since the conductivity values are too high (>20µS) and therefore the values are not useful.

The results of 160µm filtrate and 8µm filter cake sample are compared (Figure 53). Looking the Figure below, it is noticed that except for the frequency value of 200 kHz, the 8µm filter cake sample showed higher EloTrace results. As increasing the values of frequency, the strength of signal is increasingly reduced for both samples.

![Electrooptical Signal](image)

*Figure 53. Electrooptical signal for 160µm filtrate sample and 8µm filter cake sample. The comparison of these results is useful for the study. The 8µm filter cake sample showed higher results than the 160µm filtrate sample.*

Procedure 2 - Preparation of sample with sodium hexametaphosphate (SHMP) addition and ultrasonic treatment (Nettmann et al., 2013)

The EloTrace measurements for sample with sodium hexametaphosphate (SHMP) addition and ultrasonic treatment are shown in Figure below.
Figure 54. Electrooptical signal for preparation of sample with sodium hexametaphosphate (SHMP) addition and ultrasonic treatment (Nettmann et al., 2013). It is shown the relation of signal strength and frequency of electrical field.

Procedure 3 - Preparation of sample with sodium hexametaphosphate (SHMP) addition and vortex treatment (Nettmann et al., 2013)

The EloTrace measurements for sample with sodium hexametaphosphate (SHMP) addition and vortex treatment are shown in Figure below.
Figure 55. Electrooptical signal for preparation of sample with sodium hexametaphosphate (SHMP) addition and vortex treatment (Nettmann et al., 2013). It is shown the relation of signal strength and frequency of electrical field.

Procedure 4 - Preparation of sample with PBS (pH=7.2) addition and ultrasonic treatment (Nettmann et al., 2013)

The EloTrace measurements for sample with PBS (pH=7.2) addition and ultrasonic treatment are shown in Figure below.
Figure 56. Electrooptical signal for preparation of sample with PBS (pH=7.2) addition and ultrasonic treatment (Nettmann et al., 2013). It is shown the relation of signal strength and frequency of electrical field.

**Procedure 5 - Preparation of sample with PBS (pH=7.2) addition and vortex treatment (Nettmann et al., 2013)**

The EloTrace measurements for sample with PBS (pH=7.2) addition and vortex treatment are shown in Figure below.
Figure 57. Electrooptical signal for preparation of sample with PBS (pH=7.2) addition and vortex treatment (Nettmann et al., 2013). It is shown the relation of signal strength and frequency of electrical field.

Figure 58 presents all the electrooptical results for the last four procedures of biogas sample preparation.
Figure 58. Electrooptical signal for the four procedures of biogas sample preparation based on the optimized preparation of fixed samples according Nettmann et al., 2013. The blue bar represents the sample with detergent and ultrasonic treatment, the orange the sample with PBS (pH=7.2) and ultrasonic treatment, the grey the sample with detergent and vortex treatment and the yellow the sample with PBS (pH=7.2) and vortex treatment.

Unfortunately, the conductivity values for all samples are too high. Therefore, the electrooptical signals have low values and are not useful for comparison. In Figure above, it is noticed that the samples with vortex treatment have higher signal than the samples with ultrasonic treatment, but it is not so clear the influence to the results. It is observed also that while increasing the values of frequency, the strength of signal is increasingly reduced for all samples. Comparing the sample with detergent and vortex treatment with the sample without detergent and vortex treatment, the second sample shows slightly higher signals in 200 kHz and 400 kHz while the first sample has slightly higher signals in other two higher values of frequency. The samples with ultrasonic treatment have similar signal strength results in all values of frequency.

Comparing the Flow Cytometry results with electrooptical signals, it is observed that the 8μm filtrate sample from Biogas Preparation has no so useful results because of the low amount of cell in Flow Cytometry measurements. Regarding the DAPI and BOX staining, the 8μm filter cake sample has slightly higher percentages than the 160μm filtrate sample as in the EloTrace results in which the signal strength of 160μm filtrate sample is lower. The results from other four procedures based on the optimized preparation of fixed sample according Nettmann et al., 2013 are almost the same to each other and there is no great difference between Flow Cytometry and Electrooptical measurements.
Discussion, Conclusion and Outlook

Preparation of biogas samples

The application of Flow Cytometry and Electrooptical Measurements for the analysis of biogas samples requires previous sample purification due to its high content of organic and inorganic particles and the presence of cell. In order to prepare them, the first procedure that is used is easy and simple and includes only filtration steps (Biogas Preparation). Based on information from Nettmann et al., 2013, the second procedure for sample preparation is more complicated and contains also the fixation of samples. The optimized pre-treatment method includes the addition of a detergent, ultrasonic steps and filtration steps. In this study, this procedure is investigated whether the addition of detergent and the ultrasound step is necessary for purification of biogas sample. Thus, there were five different procedures to prepare the biogas samples. It is also necessary before Flow Cytometry measurements, all possible purified samples to undergo an additional preparation to avoid the clog of the capillary within the flow cytometer. The conclusion for Biogas Preparation is that the 160μm filtrate sample and the 8μm filter cake sample provide more reproducible results than the 8μm filtrate sample. The amount of cells of the 8μm filtrate sample was extremely lower than the other two and that makes it not useful for further study. Regarding the other four preparations, which are based on optimized treatment according Nettmann et al., 2013, the outcome is that the detergent is not necessary to accelerate the penetration of the fixatives into cells and to dissolve cell aggregates. The results with ultrasonic treatment are similar to the results with vortex step. Thus, this means that ultrasonic step can be replaced by the easier and lower costly treatment, vortex with same results.

Flow Cytometry measurements

Flow Cytometry is an analytical technique used in biotechnology to assess biological and physiological characteristics of several types of cells. As opposed to traditional techniques, which tend to be tedious and relatively slow, the use of Flow Cytometry reduces substantially this time. The common cytometers can measure up to thousand cells per second. It allows the analysis of large numbers of cells individually providing at line information about the culture viability and vitality. Different dyes are used, which allows the analysis of specific cellular characteristics at individual cells. There are a variety of fluorescent dyes with different properties. They have to be selected according to the purpose of the investigation. In this study, DAPI, BOX, SYTO 13 and PI are applied for staining biogas cells, but it is not used dual staining due to the fact that occurs problems of overlapping emission peaks and compensation. Therefore, these dyes are used separately with the five procedures.
In this study, the DAPI and BOX staining are further studied for the impact on physiology and morphology of biogas cells by using the Flow Cytometry technique. PI and SYTO 13 are also used in this study, but the results cannot give some useful information. PI had no influence in biogas samples. This behavior is not clear and requires further research. SYTO 13 is not useful in this study because it is needed to know the cellular viability of samples and therefore the results of BOX staining are used for comparison with Electrooptical measurements. Regarding to positive control of biogas cells, the heat shock and the addition of butanol provides reproducible results and useful for the combination with stained samples. However, a defined standard procedure is not available in literature for each type of bacteria and for different fluorescent dyes. The next step would be the on-line analysis and getting data about vitality and viability of the culture in real time and of a higher frequency will help to predict adverse effects. Therefore, the on-line measurement along with a control strategy could be used as a mean of increasing the overall process efficiency.

Staining conditions for used dyes, DAPI, BOX, SYTO 13 and PI.

According to the literature, it was not found a defined standard procedure for DAPI staining for biogas samples. Therefore, an optimized and suitable for the purposes of this study procedure was investigated. BOX, SYTO 13 and PI staining was added and performed based on protocol for *Corynebacterium glutamicum* cultivations and not by an optimization procedure. The conditions of this protocol fit equally well to the biogas samples. Moreover, the optimizing procedures for DAPI staining conditions are not often described in detail in literature. The determination of the variables and their sensitivity for the DAPI staining optimization was performed by Design of Experiments (DoE). The staining temperature, the final concentration of fluorescent dye and the staining time were determined as the variables of DAPI staining, while the response of design was the percentage of staining cells. Using a wild-type strain of genus as a model bacterium, *Clostridium acetobutylicum* ATCC 824, the optimization was done in two experimental design steps. Using these strictly anaerobic cells and with the aid of DoE, the number of necessary experiments for DAPI staining optimization was minimized. The outcome of the experimental designs was that the optimal conditions for DAPI staining are 37°C as staining temperature, 20 μg/mL as final concentration of DAPI and 30 minutes as staining time. These optimal conditions differ from the literature (Table 11). These studies have no data related to the temperature of staining, only to use the Room Temperature. The staining concentration of dye selected is significantly higher than the concentrations mentioned in bibliography data because it is considered very low for biogas samples.
Electrooptical measurements

EloTrace is the first world-wide automatic electrooptical measurement system for detection of parameters of bacterial cells. The process control achieves a balance growth and improves understanding about growth behavior and critical phases of a fermentation process. The high degree of automation also helps to determine the ideal time for harvesting with a maximize yield of stable active cells as well as increase of product quantity and quality of fermentation runs. In other words, the determination of cell activity, stress levels, cell size and morphological changes is done automatically. Using EloTrace it is measured the polarizability of five procedures which is closed linked to the actual physiological state of the biogas cell. Electrooptical measurements are done in parallel for all procedures. The signals of the 160µm filtrate sample and the 8µm filter cake sample were combined and have slight differences. The 8µm filtrate results cannot be used, since the conductivity values are too high (> 20µS) and therefore the values are not useful. The EloTrace results for the other procedures are also out of bounds because of high level of conductivity value. In future, it is needed to find out a preparation of biogas sample such that the results will be more reproducible. Comparing the Flow Cytometry results with electrooptical signals, it is observed that the 8µm filtrate sample from Biogas Preparation has no so useful results because of the low amount of cell in Flow Cytometry measurements. Regarding the DAPI and BOX staining, the 8µm filter cake sample has slightly higher percentages than the 160µm filtrate sample as in the EloTrace results in which the signal strength of 160µm filtrate sample is lower. The results from other four procedures based on the optimized preparation of fixed sample according Nettmann et al., 2013 are almost the same to each other and there is no great difference between Flow Cytometry and Electrooptical measurements. In future, it is needed to find out a preparation of biogas sample such that the results will be more reproducible.
Literature


Hellenic Biogas Association – Website of Hellenic Biogas Association (HEL.BI.O)


Invitrogen guide – Website of Invitrogen


