121 Pulsed Electric Fields-Assisted Extraction of Molecules from Bacterial and Yeast Cells

Saša Haberl Meglič

Abstract

Electroporation (*also termed as pulsed electric field–PEF*) has been used in several fields, such as clinics (electrochemotherapy, gene electrotransfer, irreversible tissue ablation, DNA vaccination); food *processing* (inactivation of microorganisms, drying, extraction of juice from fruits and vegetables); and biotechnology (bacterial electrotransformation, extraction of technologically relevant molecules from microorganisms). Later use of PEF has only started to emerge and the yields of extracted molecules are described to be the same or higher as obtained by other methods. Furthermore, several distinctive advantages over the standard techniques have been described, such as less fragmentation of microorganism and by that less purification needed; no need to use expensive and harmful chemicals and possible selective extraction of molecules. In the introduction, benefits and shortfalls of chemical and physical extraction methods are described. Subsequent sections explore in more detail *assisted* extraction of different molecules (proteins, plasmid DNA, lipids) by PEF from bacterial and yeast cells. Firstly, instantaneous plasmid DNA (pDNA) extraction/transfer from/into various species is described, where different parameters have been studied in order to obtain as much successfully transformed cells as possible. Later sections present an overview of the parameters affecting the efficiency of extraction assisted by PEF. At the end main parameters affecting the yield of extracted molecules are summarized and main challenges of PEF-assisted extraction of molecules from microorganisms are presented.

Keywords

Pulsed electric field • Electroporation • Extraction • Bacterial cells • Yeast cells • Protein • Plasmid DNA • Lipid

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Contents

Introduction 2254
Bacterial Cells 2257
Instantaneous pDNA Transfer Assisted by Pulsed Electric Field Between Bacterial Species 2257
Instantaneous pDNA Transfer Assisted by Pulsed Electric Field Between Bacterial and Yeast Cells 2261
Plasmid DNA Extraction Assisted by Pulsed Electric Field from Bacterial Cells 2262
Pulsed Electric Fields-Assisted Extraction of Proteins from Bacterial Cells 2264
Pulsed Electric Fields-Assisted Extraction of Lipids from Bacterial Cells 2266
Yeast Cells 2267
Pulsed Electric Fields-Assisted Extraction of Proteins *from Yeasts* 2267
Conclusions 2268
Cross-References 2269
References 2269

Introduction

Genetic engineering has opened a possibility to produce proteins for medicine and industry in recombinant bacteria, i.e., bacteria in which we introduce gene. The growing relevance of this field is highlighted by the fact that demands for biological molecules (e.g., proteins) and is increasing rapidly (Saccardo et al. 2016). For instance, in medicine, recombinant proteins such as human growth hormone, γ-interferon, human lysosomal enzymes, etc. are produced in microorganisms, which represent a convenient platform since they have high expression level, are fast-growing organisms, thus production time, scale, and costs are lower. Furthermore, recombinant proteins can also be used in food processing (cellulase for fermentation of biomass into biofuels), in textile industry (dissolving starches from textiles), in food industry (for food fermentation process), in biochemistry applications (horseradish peroxidase used to amplify a weak signal of a target molecule), etc. One of the most preferred and popular host systems for producing recombinant proteins is *Escherichia coli* bacteria which apart from being cost-effective, grows fast and has high protein yield. However, using *E. coli* for production of recombinant proteins can still have a few drawbacks, such as expressed proteins are accumulated within aggregates, are degraded, or their biological activity is lost during extraction or purification process (Kotnik et al. 2015).

Over the years, several routine procedures have been developed for cell cultivation in order to extract desired protein. However, particularly the cell lysis as an extraction step has been neglected, although it largely influences the subsequent purification steps. In general, extraction of proteins is achieved by chemical or physical methods (Gao et al. 2013). In Table 1, advantages and disadvantages of chemical and physical methods used for bacterial cell lysis in order to obtain proteins are summarized. Main drawbacks of many chemical methods are the use of expensive chemicals and the necessity of removing them from the final product. Furthermore, use of such chemicals in pharmaceutical industry is restricted by regulatory

Table 1 Advantages (+) and disadvantages (−) of chemical and physical methods for bacteria lysis (Naglak et al. 1990; Gao et al. 2013)

Chemical methods	+	−
Chaotropic agents (guanidine etc.)	−Weakened hydrophobic interactions in bacteria	−Ineffective in some bacteria −Acts only on growing cells
Detergents (TritonX-100 etc.)	−Effective for all bacteria −Allows selective extraction	−Affects mainly inner membrane
Solvents (chloroform etc.)	−Effective for all bacteria −Inexpensive, applicable on almost any scale −Allows selective extraction	−Possible denaturation of proteins −Necessity of using spark-proof equipment (inflammable components)
Enzymes (lysosome etc.)	−Effective for all bacteria −Highly specific action −Mild conditions needed	−High cost −Need to remove enzyme −Released nucleic acids increase the viscosity-complicated subsequent processing steps (e.g., filtration) −Different bacteria sensitivity towards various enzymes −Often limited extraction of periplasmic or "surface" proteins
Chelating agents (*ethylenediaminetetraacetic acid*-EDTA etc.)	−Allows selective extraction	−Ineffective in some bacteria
Alkali treatment	−Suitable for large-scale process −Easy application in almost any scale of operation	−Desired product must be stable at a high pH value (10.5–12.5) −Possible denaturation or degradation of proteins
Physical methods	+	−
Osmotic shock	−Allows selective extraction	−Ineffective in some bacteria −Difficulty in handling large volumes (low temperatures required for efficient operation)
Freezing and thawing	−Effective for all bacteria −High protein yield	−Non selective extraction of proteins
Wet-milling	−Effective for all bacteria	−Extensive fragmentation of bacteria −Released nucleic acids increase the viscosity-complicated subsequent processing steps (e.g., filtration) −Possible denaturation of proteins (due to the high heat), unless if cooling (for which high energy consumption is needed and therefore high cost)
High-pressure homogenization	−Effective for all bacteria −High efficiency in a single passage (compared to wet milling)	−High pressures required (1250 bars), thus heating is present (2.5 °C per 100 bar)

(*continued*)

Table 1 (continued)

Chemical methods	+	–
Sonification	–Effective for all bacteria –Fast process –Low energy and cost –Transient membrane rupture	–Can be very destructive (extensive bacteria fragmentation) –Possible protein alteration and enzyme kinetics

bodies. The largest drawback of physical methods is extensive fragmentation of bacteria, which requires a costly downstream purification process. Consequently, the cost of protein extraction remains high, providing strong motivation for new extraction tools and procedures.

In early 1950s, the first pDNA was recognized by Joshua Lederberg. By the end of the next decade, the genetic understanding of pDNA had reached a level by which massive exploitation of these genetic elements was possible. Today pDNA is used as a pharmaceutical substance in gene therapy, cell therapy, and DNA vaccination (Yarmush et al. 2014). Especially later usage of pDNA has gained recognition due to its apparent safety and simplicity compared to live-attenuated viral vaccines, since pDNA has zero threat for evolving into virulent state. Today many methods are established for pDNA isolation, but their low efficiency and/or high cost still represents a challenge. Therefore the economic perspective of this fast-developing field underlines the need for development of industrial scale method for the production of pDNA in an adequate quantity and quality.

Generally, all methods for pDNA extraction involve the disruption and lysis of cells. Choosing which method to use will depend on many selection factors including: (i) quantity of pDNA needed; (ii) purity of required pDNA; (iii) downstream applications of pDNA; (iv) time available; (v) ease of pDNA extraction technique, and (vi) expense or money available. In general, the extraction of pDNA from bacteria can be divided into three stages: bacteria disruption and cell lysis, removal of contaminants, and pDNA recovery. Bacterial cells carrying pDNA are propagated in shaker flask or fermenters and are then harvested by centrifugation. Cells in sediment are resuspended in media solution or distilled water. In order to obtain pDNA from bacteria, the cells need to be lysed. One of the most commonly used methods for lysing bacterial cells is alkaline lysis. Cells are lysed with *sodium hydroxide/sodium dodecyl sulfate* (NaOH/SDS), leading to the release of the cell content. After neutralization with acidic potassium acetate, pDNA remains in solution, while denatured proteins, genomic DNA, and cellular debris are coprecipitated in insoluble salt-detergent complexes. Precipitated debris is removed by either centrifugation or filtration, producing cleared lysates, from which pDNA is purified. Alkaline lysis on large scale production has several drawbacks such as increase of the volume, preparation of different solutions, and entrapment of pDNA in cells debris with subsequent lower recovery of pDNA which then requires introduction of additional extraction steps. Consequently the cost of pDNA manufacturing remains quite high and the need to develop new pDNA isolation tools in order to lower production cost is evident (Haberl et al. 2013).

Almost four decades ago, a physical method – electroporation – which resulted in significant increase of the cell plasma membrane permeability for different molecules was described (Kotnik 2016b). Namely, when electric field is applied to a biological cell, transmembrane voltage is induced. When induced transmembrane voltage exceeds a certain value, structural rearrangements of lipids in the membrane bilayer occurs. As a consequence membrane becomes permeable and small or large molecules that otherwise cannot pass the cell membrane can be introduced into or extracted from cells. Therefore electroporation has been used in several fields, one of them being also assisted extraction of biomolecules by pulsed electric fields (PEF) from several microorganisms (Haberl Meglic and Kotnik 2016).

Subsequent sections “Instantaneous pDNA Transfer Assisted by Pulsed Electric Field Between Bacterial Species” and “Instantaneous pDNA Transfer Assisted by Pulsed Electric Field Between Bacterial and Yeast Cells” describe first attempts of assisted-*pDNA* extraction by PEF from microorganism cells where instantaneous extraction and transformation occurs. Later sections describe extraction of various biomolecules from different microorganism species.

Bacterial Cells

In microbiology, electroporation was most commonly used for the genetic transformation of bacteria – to stimulate uptake of pDNA. At first reported transformation efficiencies ranged from 10^2 to 10^4 transformants per μg of pDNA. Later with the optimization of electroporation parameters, the efficiency reached more than 10^9 transformants per μg of pDNA. Since electroporation was found to be simple and rapid, allowing transformation of pDNA to be accomplished in minutes, the idea aroused to also extract intact pDNA from bacterial cells (Garcia and Buie 2016).

Instantaneous pDNA Transfer Assisted by Pulsed Electric Field Between Bacterial Species

Since 1946 it was known that bacteria of different strains can exchange genetic material by three different natural mechanisms (conjugation, natural competence for DNA uptake, and viral transduction). Also a fourth natural mechanism of genetic exchange, triggered by atmospheric electrostatic discharges (lightning), describes a possible role of electroporation in bacterial evolution (Kotnik 2013, 2016a).

Today electroporation in laboratories is most commonly used in transformation experiments in order to stimulate pDNA uptake into bacterial cell. Also some microorganisms, which were difficult to transform by established procedures, have been successfully transformed by electroporation. Therefore in early 1990s, researchers started to wonder if pDNA can also be transferred directly by electroporation from a pDNA-bearing to a pDNA-free strain of bacteria (Fig. 1).

Stationary phase donor and recipient bacteria cultures of Gram-negative bacteria *E. coli* were mixed together and transferred to a chilled cuvette. Donor strain carried

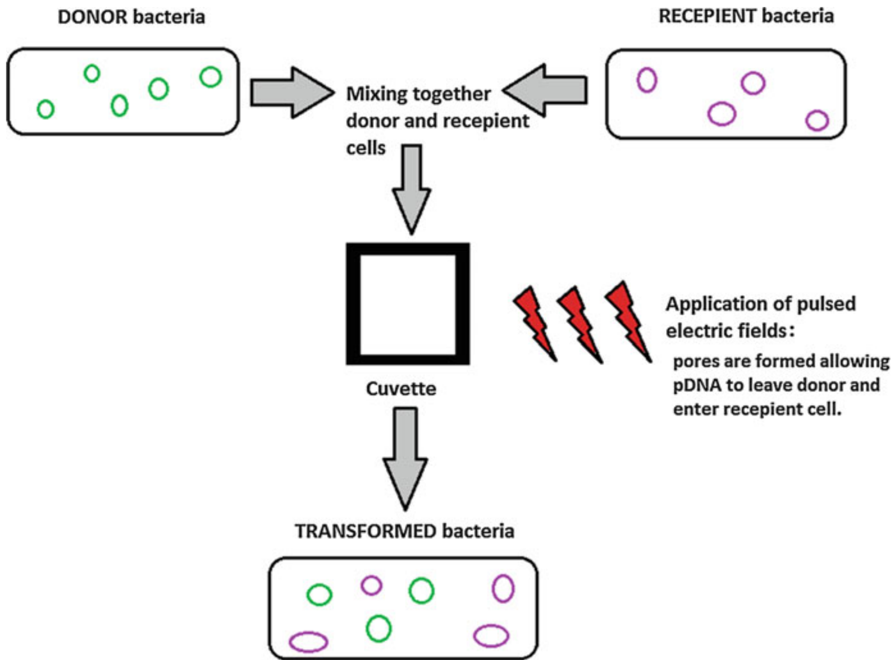

Fig. 1 Instantaneous pDNA transfer assisted by pulsed electric field between bacterial species. Transformed bacteria is carrying pDNA from donor and recipient bacteria (Image by the author, not published previously)

pDNA which ensured tetracycline resistance, while recipient strain carried pDNA with kanamycin resistance. A single pulse was applied with amplitude 2.4 kV (electric field between the electrodes being 12 kV/cm) and 25 μF capacitance (with time constant of 4.6 ms). After applying electric pulse, a *super optimal broth with catabolite repression* (SOC) medium was added to ensure bacterial viability after electric field application. Afterwards bacterial cells were incubated for 1 h at 37 °C to ensure pDNA to be transformed through the pores in recipient bacterial membrane developed by pulsing. Per electroporation approximately 3×10^3 colonies grew on kanamycin and tetracycline agar plates, meaning that pDNA carrying tetracycline resistance in donor cells was instantaneously extracted and transformed into recipient bacterial cells, which carried only kanamycin resistance. The method was validated also by instantaneous PEF transfer of pDNA carrying ampicillin resistance, where around 2×10^5 colonies were detected on selection agar plates. Authors concluded that since PEF mechanism is solely physical (not biological), it has the potential to transfer nonconjugative pDNA between *E. coli* and other species (Summers and Withers 1990).

The same year Pfau and Youderian (1990) showed that instantaneous pDNA transfer assisted by PEF was more efficient between bacterial cells in an exponential growth phase than between cells in stationary phase. Authors demonstrated successful pDNA transfer between two Gram-negative species – *Escherichia coli* and

Salmonella typhimurium – adding a single pulse. They also reported that optimum PEF conditions depended on the direction of transfer and that natural electrical discharges (such as lightning) may facilitate lateral transfer of genetic material between species, which was also in detail described by Kotnik (Kotnik 2016a).

Successful instantaneous pDNA transfer assisted by PEF was shown also for several different *E. coli* species. Different pDNA sizes (2.7–21.7 kbp) and PEF conditions were tested: electric field between the electrodes ranging from 6.25 to 12.5 kV/cm, capacitance 0.25–25 μF (with time constant ranging from 0.9 to 5.5 ms), and pulse number from 1 to 20. Three different techniques were employed in order to instantaneously transfer pDNA from donor to recipient cells: (i) donor cells were subjected to PEF, centrifuged, and the supernatant served as a source of pDNA, which was added to recipient cells ("pulse-spin-pulse" technique). Again PEF was applied to transform pDNA from the supernatant into recipient cells; (ii) PEF was applied to donor cells, recipient cells were added, and again PEF was applied; and (iii) a mixture of donor and recipient cells were subjected to PEF together. The efficiency of instantaneous pDNA transfer between *E. coli* species ranged from 0 to 3.2×10^4 per μg of pDNA. It was largely depended on different parameters: (i) PEF conditions – higher electric field resulted in higher transformation efficiency; (ii) bacterial growth phase – cells in exponential growth phase transformed at the highest frequency. Authors stressed out that when studying transfer of pDNA between two bacterial strains, it is important to take into account the growth phase of both donor and recipient cells; (iii) technique used to transfer pDNA from donor to recipient cells – best being "pulse-spin-pulse" technique; (iv) pDNA size – the number of successfully transformed cells decreased as the pDNA size increased. This observation was also found in conventional transformation techniques. Furthermore, successful instantaneous pDNA transfer assisted by PEF between two different Gram-negative bacterial species was shown – pDNA was transferred from *E. coli* into *Pseudomonas aeruginosa* or *Salmonella typhimurium*. Authors concluded that PEF method can be used as a rapid and convenient technique to extract pDNA from one species and transfected it into another. Furthermore, they suggested that PEF should be used as a way to transfer other molecules (such as bacteriophages, ribonucleic acid-RNA molecules, or other cellular components) from one cell population to another (Kilbane and Bielaga 1991).

The same year, the first report of instantaneous successful transfer assisted by PEF of pDNA from donor Gram-positive bacteria (*Lactococcus lactis*) to recipient Gram-negative bacteria (*Escherichia coli*) and vice versa was demonstrated (Ward and Jarvis 1991). It has to be pointed out that the envelope of Gram-positive bacteria is constructed differently than the one of Gram-negative bacteria (Fig. 2) and because of that it acts differently in electric field.

Namely, it was shown that Gram-positive bacterial species are more resistant to electroporation, due to their thick peptidoglycan layer (Fig. 2). In a study of Ward and Jarvis, donor *cells* (*L. lactis* or *E. coli)* were grown to the stationary growth phase and subjected to the optimum pulse for the donor strain (*L. lactis*: 12.5 kV/cm, 25 μF, 100 Ω parallel resistance and duration of the pulse through the cells was 2.4 ms; *E. coli*: 12.5 kV/cm, 25 μF, 800 Ω parallel resistance and duration of the pulse

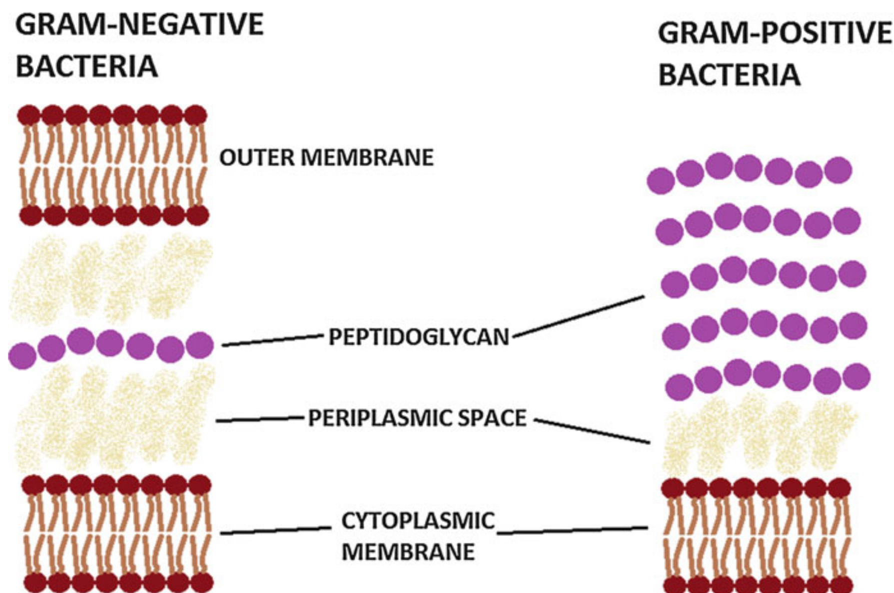

Fig. 2 Simplified structure of the envelope of Gram-negative and Gram-positive bacteria (Zerbib et al. 2016) (Image by the author, not published previously)

through the cells ranged from 15 to 15.6 ms). Donor cells were centrifuged in order to remove bacterial cells and the supernatant (where was the extracted pDNA from donor cells) was again mixed together with recipient cells and subjected to the optimum pulse as described above. After applying electric pulse, a rich medium appropriate for recipient bacterial cells was added to ensure bacterial viability after electric field application. Transfer of the pDNA was verified by a rapid pDNA preparation from a culture grown from a single colony on the selective media. The transfer efficiency was slightly better when *E. coli* cells were used as donor cells (1×10^3 transformants per electroporation) compared to when *L. lactis* were used as donors (1×10^2 transformants per electroporation). The results could not be directly compared to other techniques as authors did not purified pDNA from donor cells, therefore pDNA concentration was not determined. Authors concluded that optimum growth phase for recipient cells should be exponential, while stationary growth phase is adequate for donor cells. In contrast to study of Kilbane and Bielaga (1991), washing of cells before pulsing was necessary in order to prevent arcing after which no or little transformation was observed.

In order to overcome difficulties of standard pDNA extraction techniques from *Mycobacterium* spp., such as high resistance of bacterial cells to standard cell lysis in addition to low pDNA copy numbers, an instantaneous pDNA transfer assisted by PEF between two distant bacterial species was described: *E. coli* (phylum Proteobacteria) and *Mycobacterium* spp. (phylum Actinobacteria) (Baulard et al. 1992). Namely, *E. coli-Mycobacterium* shuttle pDNAs are commonly used to take

Table 2 Time frame for transformation and pDNA analysis of *Mycobacterium* spp. – standard protocols versus electroporation protocol (Summarized from Baulard et al. 1992)

	M. smegmatis	*M. bovis*
Time needed when **PEF** is used	8 days	15–17 days
Time needed when **standard protocol** is used	14–17 days	29–34 days

full advantage of the molecular biological tools developed in *E. coli*, before transferring final pDNA into *Mycobacterium* spp. For the first time authors reported instantaneous pDNA transfer assisted by PEF between very distant bacteria species. *Mycobacterium smegmatis* or *Mycobacterium bovis* were used as donor cells and *E. coli* as a recipient. A single pulse was used to transfer pDNA from *Mycobacterium* spp. into *E. coli* cells, with pulse amplitude of 2500 V (electric field strength cannot be determined, since no distance between the electrodes was described) and 45 μF. Instantaneous pDNA transfer assisted by PEF between *M. bovis* and *E. coli* resulted in 10–100 transformants per *M. bovis* colony and 10^4 transformants per *M. smegmatis* colony. Authors concluded that this procedure is applicable especially for slow growing or fastidious bacteria or for bacteria that are resistant to standard cell lysis. In Table 2 the advantage of fast, instantaneous pDNA transfer assisted by PEF compared to slow standard protocols was shown.

Instantaneous pDNA Transfer Assisted by Pulsed Electric Field Between Bacterial and Yeast Cells

All reports described above studied pDNA extraction *assisted* by PEF from bacterial cells and its direct transfection also into bacterial cells. Only few studies in early and mid-1990s showed that instantaneous pDNA transfer assisted by PEF between bacterial and yeast cells is also possible. Namely, pDNA is easily purified from *E. coli* bacteria, therefore pDNA in yeast are often transferred back to *E. coli* for detailed characterization. Kilbane and Bielaga were first to show that simultaneous pulsing of donor (*E. coli*) and recipient (*S. cerevisiae*) cells resulted in successful transfer of pDNA. Nevertheless, authors could not accurately determine the frequencies of pDNA transfers (Kilbane and Bielaga 1991).

Marcil and Higgins showed that an instantaneous pDNA transfer assisted by PEF from *S. cerevisiae* to *E. coli* is also possible. Their motivation was that *S. cerevisiae* can be transformed with a pDNA in order to study or identify sequences of interest on pDNA. Afterwards these pDNA are retrieved out of *S. cerevisiae* and are transfered back into *E. coli* in order to additionally analyze it or manipulate it. However, the retrieval of pDNA from *S. cerevisiae* is quite time consuming and cumbersome. Therefore, authors subjected *S. cerevisiae* harboring pDNA (size of 5.6 kB) to PEF together with *E. coli* cells. PEF was applied two times with protocol as follows: (i) mixing together yeast *S. cerevisiae* of different strains with *E. coli* bacteria; (ii) first pulsing – 7.5 kV/cm, 50 μF, and 100 Ω (pulses optimal for yeast); (iii) chilling the mixture for 30–60 s; (iv) second pulsing – 1.5 kV/cm, 50 μF, and

150 Ω (pulses optimal for *E. coli*); and (v) adding SOC medium and incubating cells for 1 h at 37 °C. The transformation efficiency was 600–1000 transformants per yeast colony (Marcil and Higgins 1992).

A few years later, a rapid transfer of low-copy number episomal pDNA assisted by PEF from *S. cerevisiae* to *E. coli* was described. Researchers tried the same procedure as described by Marcil and Higgins (1992), which yielded only few transformants (maximal 2). The proposed reason was that inefficient extraction assisted by PEF from yeast cells occurred or that *E. coli* cells did not survive two pulsing. Therefore yeast cells were treated with other techniques (glass bead grinding, heating at 100 °C, or freezing/thawing), afterwards mixed together with competent *E. coli* cells and subjected to PEF. In some experiments also detergent Triton X-100 was added to yeast cells in order to increase cell membrane disruption of yeast cells. The highest number of transformants was obtained when yeast cells were treated with glass beads and subjected to heating at 100 °C for 1 min. When *E. coli* cells were added, 12.5 kV/cm of electric field was applied with 25 μF, 200 Ω, and 4.3 ms of time constant. Authors concluded that although this procedure is not as fast as instantaneous pDNA transfer assisted by PEF and not as successful as using pure yeast pDNA, it still requires few days less time and is well suited for applications where only few transformants are needed (Gunn and Nickoloff 1995).

Plasmid DNA Extraction Assisted by Pulsed Electric Field from Bacterial Cells

Only few studies up to now showed *assisted* extraction of pDNA from bacterial cells by PEF. Calvin and Hanawalt (1988) were the first to test the possibility that pDNA *extraction from bacterial cells could be assisted by PEF* and quantified the results on agarose gel. An overnight culture of *E. coli* in a stationary growth phase was subjected to different number of electric pulses of different electric field strengths. The amount of extracted pDNA was determined by relative intensity of the band on the agarose gel, compared with the band intensity of a known amount of pDNA run in a parallel lane on the same gel. From a 2 ml of bacterial culture about 5 μg of pDNA was extracted, which was close to the yield obtained by alkaline method. By adding number of pulses or increasing electric field strength, pDNA yield increased, but also genomic DNA and RNA were released by an even larger factor. Therefore, pulse parameters need to be optimized in order to extract as pure pDNA as possible while releasing relatively small quantities of genomic DNA and RNA molecules.

Rapid pDNA extraction assisted by PEF from small liquid cultures or single colonies of cyanobacteria *Nostoc* or *Anabaena* was shown a few years later (Moser et al. 1995). Cyanobacterial cells were after growth suspended in a media and pulsed once (25 μF capacitance, 200 Ω resistance, and time constant approximately 4.5 ms). DNA concentrations were determined by spectrophotometry or by comparison of band intensities on stained agarose gels. The amount of material released from cyanobacterial cells depended on different factors: (i) electric field strength, (ii) growth conditions and treatment after the pulse:

(i) At electric field strength between 12 kV/cm and 20 kV/cm greater release of nucleic acid was observed, especially covalently closed forms of pDNA were extracted. When cyanobacterial cells were subjected to electric field strength of 20 kV/cm and higher, less compact linear or open circular pDNA were extracted with also some genomic DNA fragments. Authors used extract to also transform *E. coli* cells in order to show that in the sample extracted pDNA was present and it was able to transform other bacterial species lacking this pDNA.

(ii) From stationary cells grown under continuous light immediate release of phycobiliproteins and nucleic acids was observed after pulse delivery. On contrary, cyanobacteria grown in light–dark cycles required incubation of up to 4 h at 4 °C after pulse delivery in order to promote leakage of cellular content.

Under appropriate conditions (higher electric field strength and 4 h incubation at 4 °C after the treatment), the yield of extracted pDNA – the size of which was 9.7 kb – from 10^8 *Nostoc* cells (5 ml of culture) was approximately 2 μg, which is comparable to other methods. Authors also examined the possibility to extract from cyanobacteria sufficient amount of genomic DNA for *polymerase chain reaction* (PCR) amplification. Their conclusion was that extraction assisted by PEF is applicable for the recovery of recombinant pDNA and genomic DNA for PCR amplification and also other macromolecules from a variety of cyanobacteria from which such extraction have previously been cumbersome.

In a study by Takayuki et al. (1999), different electroporation media were tested for extraction of pDNA from *E. coli* cells: distilled water, TE media (Tris and EDTA media used in molecular biology to solubilize DNA, while protecting it from degradation), and physiological saline solution. When TE media was used, relatively large amounts of nucleic acid molecules were extracted, when 6.5 kV/cm of electric field strength was applied with 60 s of pulse treatment. Nevertheless, pDNA could not be extracted with selectivity, since others kinds of nucleic acids (genomic DNA, RNA) were present in final sample. For that reason also RNase enzyme was used in TE media, but after the treatment authors could not detect the significant release of nucleic acid molecules. Their conclusion was that pDNA extraction assisted by PEF is possible, but it is difficult to purify the pDNA simultaneously.

Only after 14 years, a study compared two methods for pDNA recovery: extraction assisted by PEF and alkaline lysis (Haberl et al. 2013). For the first time, square wave pulses (instead of exponentially decaying) with different duration and electric field strength were used to extract pDNA from *E. coli* cells. For alkaline lysis method, two lysis incubation times were performed: 5- or 10-min. Extracted pDNA from both methods was quantified by anion exchange chromatography. No difference was observed in pDNA concentration when the 5- or 10-min lysis time was used in alkaline lysis method; therefore, all bacterial cells were already disrupted after 5 min of lysis. Different electric parameters regulate the degree of bacterial membrane permeabilization and thus control the release of intracellular content (proteins, pDNA, etc.); therefore, optimal pulse parameters were determined for *E. coli* cells: the highest amount of extracted pDNA was obtained when longer millisecond pulses were applied, with lower electric field strength (one pulse of 1 ms

duration and 12.5 kV/cm of electric field strength). At those conditions, threefold higher concentration of pDNA compared to 10-min alkaline lysis was obtained. Since electric pulses could harm the functionality of pDNA, transformation of *E. coli* with pDNA, extracted by PEF, was performed, where the highest transformation efficiency was obtained with pDNA extracted with one rectangular pulse with a duration of 1 ms and an applied voltage of 1,250 V, resulting in $E = 12.5$ kV/cm. Authors concluded that the main advantages of extracting pDNA by PEF compared to alkaline lysis are higher pDNA yield, shorter process time, and reduced lysate volume.

During recent years, advances in micro- and nano-scale techniques for various analytical and preparative procedures have been remarkably rapid, thus recent study used microfluidics to control the degree of cell opening to obtain a targeted release of biomolecules from the cells (Matos et al. 2013). Nucleic acids extracted from *E. coli* cells were examined and quantified using agarose gel and nanophotometer. The electrical field between the facing electrodes in a microchip was applied via a DC power supply and even at lower voltages (below 0.5 V) high levels of nucleic acids could be detected (mainly low molecular weight RNA molecules). For larger nucleic acids such as pDNA, the formed pores need to be larger and opened for extended time periods, which was impossible without compromising the viability of the cells. Extraction of pDNA was thus only possible at higher applied voltages, i.e., at 1–2 V, where partial to complete cell lysis occurred.

In summary in all reviewed reports, authors successfully extracted pDNA by PEF from bacterial cells. Although it was difficult to compare the yield, since pulses applied are of exponentially decaying or square wave form and not all pulse parameters are presented in studies (i.e., electric field strength, pulse number, pulse duration).

Pulsed Electric Fields-Assisted Extraction of Proteins from Bacterial Cells

One of the promising methods for extracting proteins from cells was found to be PEF treatment. Namely, some intracellular proteins could be released through the pores induced by PEF with selectivity, because the size of these pore area can be controlled according to the electric parameters. Furthermore, with adjusting electric field, proteins accumulated only in periplasmic space of Gram-negative bacteria can be extracted (Fig. 3).

The effect of different parameters of PEF treatment (electrode shape, electroporation media and electric parameters) on extraction of three recombinant proteins (β-glucosidase, cellobiohydrolase and α-amylase) from *E. coli* was studied (Ohshima et al. 2000). In a chamber where an un-uniform electric field was applied (the strongest being around the needle electrode) the highest amount of all three studied proteins was extracted. When 5% of glycine was added (it increases the permeability of cell membrane), the amount of protein which is mainly accumulated in cytoplasm (β-glucosidase) increases. Nevertheless, the opposite effect was observed for proteins which are mainly present in periplasmic space (e.g.,

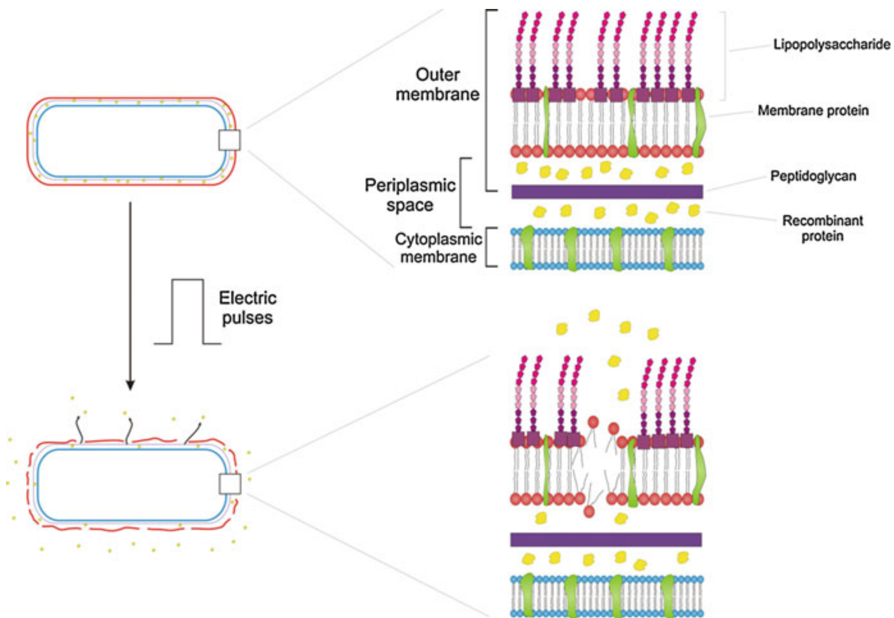

Fig. 3 Cartoon of extracting periplasmic recombinant proteins by PEF treatment. If PEF parameters are adjusted, a selective permeabilization of bacterial outer membrane is possible, thus recombinant proteins accumulated in periplasmic space could be released out of bacteria (Image by the author, not published previously)

cellobiohydrolase and α-amylase). By adding polyethylene glycol (PEG) (which should stabilize the pore in the membrane formed by PEF) or *sodium chloride* (NaCl), total protein concentration gradually increases. Nonetheless, PEG or NaCl concentration should be optimized if selective protein release is needed, since the dependence of PEG or NaCl on protein release is different for each protein.

To improve the productivity of recombinant protein, high cell density cultivation using the fed-batch cultivation technique has been proposed where an extraction of recombinant α-amylase from *E. coli* by PEF was reported (Shiina et al. 2007). If bacterial cells during their growth were subjected to continuous PEF treatment, only a small amount of α-amylase was extracted due to the fact that PEF caused decrease in cell growth. But if cells in stationary growth phase were subjected to PEF intermittently, the concentration of extracted α-amylase was about 30% of the total amount of this protein produced in bacteria. By SDS-*polyacrylamide gel electrophoresis* (SDS-PAGE) and activity staining, authors also proved that extracted α-amylase by PEF treatment was not digested and was active.

Recently, the influence of several parameters on protein extraction from *E. coli* cells by PEF was also studied: (i) pulse parameters, (ii) temperature, (iii) bacterial growth phase, (iv) incubation media, and (v) bacterial strain (Coustets et al. 2015; Haberl Meglic et al. 2015; Haberl-Meglič et al. 2016).

(i) Since electrical pulse parameters are affecting cell membrane permeability and undesired membrane contaminants, such as endotoxins could be released from the outer membrane of bacteria cells, pulse treatment conditions have to be adjusted in order to extract a maximum quantity of proteins by PEF, with high cell viability. The influence of different electric parameters – electric field strength, pulse duration, pulse number, pulse frequency and energy input – have been studied on protein extraction efficiency by PEF (Haberl Meglic et al. 2015). The highest amount of proteins was extracted by PEF when higher electric field strength or longer pulse duration was applied. No statistically significant effect was observed for different pulse number or pulse frequency. Furthermore, also the energy input seems not to directly correlate with yield of extracted proteins. Namely, at the highest energy input, authors did not obtain also the highest protein concentration (Haberl Meglic et al. 2015).

(ii) Temperature has a significant effect on cell membrane structure and by that on permeabilization of the cell membrane caused by PEF. It was also shown that post-pulse incubation at lower temperatures (at 4 °C) increases concentration of extracted proteins by PEF from bacterial cells, since its membrane does not reseal as quickly after PEF as it would at higher temperatures (Haberl-Meglič et al. 2016).

(iii) Bacterial growth phase strongly affects bacterial metabolism and also cell wall structure and porosity. Therefore, bacterial cells at different growth phases respond differently to electroporation. The highest amount of proteins are extracted by PEF from cells in middle exponential phase, while in stationary growth phase almost no effect of PEF on protein extraction was observed (Coustets et al. 2015). Nevertheless, in another study the same amount of proteins were extracted by PEF, when bacterial cells were in exponential or stationary growth phase (Haberl-Meglič et al. 2016). The difference could be due to different parameters (temperature, electric pulses) or bacteria used.

(iv) For protein recovery by PEF, it is crucial in which media bacterial cells are incubated after pulsing. In media containing EDTA and dithiothreitol (DTT) considerable increase in protein-*assisted* extraction by PEF was shown (Coustets et al. 2015).

(v) Different bacterial strains of the same species can have different sensitivity to electric field. Thus, for each bacterial strain electric pulse parameters, bacterial growth phase and incubation media after PEF should be determined in order to extract maximum amount of proteins by PEF (Coustets et al. 2015).

Pulsed Electric Fields-Assisted Extraction of Lipids from Bacterial Cells

Cyanobacteria *Synechocystis* was used as a feedstock of nonpetroleum-based diesel fuel – intracellular lipids were extracted by PEF technology. Since treatment with only PEF caused significant damage to cells, which lead to biomass loss, low-toxicity solvent isopropanol was used after milder PEF treatment. Thus, PEF

was shown to lower the costs and environmental burden (due to less isopropanol used) of lipid extraction from cyanobacteria cells (Sheng et al. 2011).

Yeast Cells

Yeasts are eukaryotic microorganisms belonging to fungus kingdom which are widely used as hosts for an industrial production of proteins, and they have a great potential being a host for foreign genes derived from higher animals. Extraction from yeasts assisted by PEF was first used for transfer of their DNA into recipient bacteria. Soon afterwards extraction assisted by PEF of different proteins (alcohol dehydrogenase, 3-phosphoglycerate kinase, glutathione reductase, hexokinase, glyceraldehyde-3-phosphate dehydrogenase, β-D-galactosidase) in a batch or flow system from various yeast species – *Saccharomyces cerevisiae, Kluyveromyces lactis*, and *Schizosaccharomyces pombe* – was described. *PEF-assisted extraction of proteins showed that the specific activities of such proteins* are higher than those obtained by mechanical disintegration or enzymatic lysis. Furthermore, PEF treatment preserved to a large extent the intactness of the yeast vacuoles, as was shown by the low leakage of proteolytic enzymes.

It was also shown by scanning electron microscopy that PEF not only permeabilizes yeast membrane but also changes its wall structure, which leads to increased wall porosity (Ganeva et al. 2013).

Pulsed Electric Fields-Assisted Extraction of Proteins *from Yeasts*

In middle 1990s, a bear yeast *S. cerevisiae* and baker's yeast were used to extract proteins by PEF. Authors used exponentially decaying pulses and noted that by increasing electric field strength (up to 25 kV/cm), protein concentration also increases to maximum 40 μg/ml (which was about 30% of the amount obtained with glass bead homogenization). Nevertheless, at those conditions cell viability significantly dropped. Authors also noted that by adjusting electric field strength, selective release of intracellular proteins can be achieved. Namely, at 6 kV/cm mostly invertase protein (its location being around the cell membrane) was extracted and at 12 kV/cm mainly alcohol dehydrogenase protein (its location being in a cytoplasm near the center of the cell) was extracted. The yield of extracted proteins by PEF also depended on yeast species – due to different thickness of cell wall – one species being more resistant to electric pulses than other (Ohshima et al. 1995).

Later several parameters have been studied, which influenced the yield of extracted proteins from diverse yeast species by PEF, when rectangular pulses are applied: (i) media, (ii) time after PEF needed to obtain maximum amount of proteins, (iii) growth phase of yeast cells, and (iv) electric parameters.

(i) The presence of potassium or sodium chloride in post-pulse incubation media provoked an accelerated release of proteins from *S. cerevisiae* cells. For yeast

S. pombe, an application of electric field in hypertonic media (1.5–2.5 M sorbitol) yielded approximately three times more proteins compared to isotonic or hypotonic media. Authors hypothesized that long exposure of cells to hyperosmotic stress after PEF accelerates irreversible membrane damage, thus increases protein extraction from cells (Suga and Hatakeyama 2009). For some yeast species by adding DTT after PEF accelerated release of proteins can be observed (Ganeva et al. 2013).

(ii) Yeast cells need to be incubated after PEF for several hours in order to allow protein efflux from cells. The most optimal time needs to be determined for each yeast species and each protein separately, i.e., for *S. cerevisiae* the most optimal incubation times determined were between 4 and 10 h (4 h for 3-phosphoglycerate kinase, 6 h for alcohol dehydrogenase, and 8–10 h for glutathione reductase) and for *K. lactis* around 4 h after PEF.

(iii) It was found that transition from middle to late exponential growth phase decreases the yield of extracted proteins by PEF. Namely, it was shown for *S. cerevisiae*, that the yield of 3-phosphoglycerate kinase, glutathione reductase, or alcohol dehydrogenase decreases by 2-, 3.4-, or 48-times, respectively, if cell are pulsed in late exponential growth phase.

(iv) Yeast membrane permeabilization and associated protein extraction by PEF are greatly depended on electric field strength and/or pulse duration. Between these two electrical parameters exists a correlation – meaning if shorter pulses are applied, higher electric field is required to obtain the same degree of structural membrane rearrangement and also vice versa. It was shown for yeasts *S. cerevisiae*, *S. pombe*, and *K. lactis* in a flow through system that by increasing electric field strength from 2.3 to 4.17 kV/cm, the yield of extracted proteins by PEF also increases (Ganeva et al. 2013).

When combining PEF treatment with high-pressure homogenization technique, improvement of yeast disruption was observed, which can have a good potential in biotechnological and food applications.

Conclusions

Pulsed electric field-assisted extraction of proteins from bacterial cells shows great promise, since it is quick (few seconds time-scale), reproducible, can be used either in a batch or continuous mode, and is therefore easily implemented into existing production lines (Haberl-Meglič et al. 2016). In order to optimize the protocol, however, several parameters need to be considered for each microorganism species separately, such as electric pulse parameters, growth phase, temperature, electroporation media, treatment after pulsed electric field, etc. Mainly by increasing electric field strength, the yield of extracted molecules rises, though one needs to be careful, since too high electric field results also in total detrimental of microorganism. Namely, by total disintegration other unwanted molecules are present in final sample, therefore need for purification is evident. It was also found that mainly

exponential growth phase is more favorable for pulsed electric field-assisted extraction, since the ratio of protein to lipid content in microorganism envelope through growth changes. Thus when the cells are in stationary growth phase, the electric field was found to be completely inefficient. But this finding was also microorganism's strain dependent.

All issues outlined above point to a need for determining most favorable parameters for each species separately. Furthermore, deeper understanding of pulsed electric field's effect on microorganism's membrane and cell wall is needed.

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Cross-References

► Bacterial Cell Envelopes: Composition, Architecture, and Origin
► Electroporation-Based Applications in Biotechnology
► Lightning-Triggered Electroporation as a Mechanism for Horizontal Gene Transfer
► Transmembrane Voltage Induced by Applied Electric Fields

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