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Protein Extraction by Means of Electroporation from *E. coli* with Preserved Viability

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Abstract Extracting proteins by means of electroporation from different microorganisms is gaining on its importance, as electroporation is a quick, chemical-free, and cost-effective method. Since complete cell destruction (to obtain proteins) necessitates additional work, and cost of purifying the end-product is high, pulses have to be adjusted in order to prevent total disintegration. Namely, total disintegration of the cell releases bacterial membrane contaminants in the final sample. Therefore, our goal was to study different electric pulse parameters in order to extract as much proteins as possible from *E. coli* bacteria, while preserving bacterial viability. Our results show that by increasing electric field strength the concentration of extracted proteins increases and viability reduces. The correlation is reasonable, since high electric field destroys bacterial envelope, releasing all intracellular components into surrounding media. The strong correlation was also found with pulse duration. However, at longer pulses we obtained more proteins, while bacterial viability was not as much affected. Pulse number and/or pulse repetition frequency at our conditions have no or little effect on concentration of extracted proteins and/or bacterial viability. We can conclude that the most promising pulse protocol for protein extraction by means of electroporation based on our experience would be longer pulses with lower pulse amplitude assuring high protein yield and low effect on bacterial viability.

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Introduction

Proteins represent an essential part of each biological cell where they participate in virtually every cell process (Cooper 2000). Production of these biomolecules in diverse cells (e.g., recombinant bacterial cells, microalgae, yeasts, etc.) has opened an important field in food industry, medicine, and pharmacy (Olempska-Beer et al. 2006; Assenberg et al. 2013). In food industry, enzymes such as amylase or cellulase can be used in food fermentation process (bread making, brewing beer, and liquor made from sugars derived from starch) (Gurung et al. 2013), in textile industry (to dissolve starches from fabrics) (Gurung et al. 2013) and in laundry or dishwasher detergents (Niyonzima and More 2014). In pharmacy, cellulase can also be used in fermentation of biomass into biofuels (Assenberg et al. 2013). Also some proteins produced in recombinant bacterial cells can be of a great value in medical applications such as human growth hormone, which can be used as a replacement therapy or γ-interferon for treatment of viral or malignant diseases (Kargi and Merriam 2013; Roff et al. 2014; Schiavoni et al. 2013).

Today a variety of techniques are known and are used to disrupt cells in order to harvest desired intracellular products (e.g., proteins), such as chemical (using solvents, detergents, alkali, or acids), biological (e.g., enzymatic lysis), or physical methods (e.g., sonification, ultrasound, high-pressure homogenization, and glass bead homogenization) (Schütte and Kula 1990; Geciova et al. 2002). Nevertheless, these methods face problems such as low recovery of targeted molecule, usage of undesired

chemicals in the process, high cost, time consumption, and high level of cellular debris, which make the downstream separation process difficult, time consuming, and expensive (Schütte and Kula 1990; Geciova et al. 2002).

In early 1970s, a physical method (electroporation) was described, where the permeability changes were induced by electric pulses (Neumann and Rosenheck 1972). Additional research in this field showed that when a biological membrane is exposed to electrical pulses of sufficient strength, transmembrane voltage exceeds a certain value and cell membrane becomes transiently permeable (Kotnik et al. 2010). Therefore, since 1980s electroporation gained ground as a tool for introducing small or large molecules into cells: foreign genes (gene electrotransfer) (Neumann et al. 1982; Wong and Neumann 1982; Daud et al. 2008) and membrane-impermeant anti-cancer drugs (electrochemotherapy) (Okino and Mohri 1987; Miklavcic et al. 2014). Recently, electroporation showed also a great potential for extracting a variety of molecules from different microorganisms: oil from microalgae (Flisar et al. 2014), proteins from microalgae (Coustets et al. 2013; Matos et al. 2013), bacteria (Ohshima et al. 2000; Shiina et al. 2007; Matos et al. 2013), yeast (Ganeva et al. 2003; Suga et al. 2007; Suga and Hatakeyama 2009), and nucleic acids from bacterial cell (Haberl et al. 2013a; Matos et al. 2013). Based on this research, electroporation's advantages compared to other extraction techniques are considered to be shorter process time (in a microsecond to millisecond range), no need for additional procedures to obtain targeted molecule and/or adding undesired chemicals into product.

Since electrical pulse parameters are affecting cell membrane permeability (Rols and Teissie 1990; Pucihar et al. 2011) 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 intracellular product by means of electroporation, with high cell viability. Namely, membrane contaminants, such as endotoxins could be released from damaged membrane, and additional purification steps are needed, which at large scales would represent up to 80 % of the production costs. Bacterial endotoxins (also known as lipopolysaccharides) are part of the outer membrane of gram-negative bacteria (for example *E. coli* bacteria) and are undesired molecules in the sample, since they elicit strong immune response in mammals. The purpose of this study was to explore different electric pulse parameters in order to extract proteins from *E. coli* bacteria by means of electroporation, while preserving as much as possible cell viability. Hence total released protein concentration and bacterial inactivation were determined in the same experiments.

Materials and Methods

Bacterial Cell Preparation

Escherichia coli K12 TOP10 strain bearing plasmid pEGFP-N1, which encodes kanamycin resistance (Clontech Laboratories Inc., Mountain View, CA, USA) was used in this study. Bacterial cells were grown in Luria Broth medium (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) containing 50 µg/ml of antibiotic kanamycin sulfate (Carl ROTH Gmbh, Essen, Germany) for 17 h by shaking at 37 °C. Cell's pellet was collected by centrifugation (4248×*g*, 30 min, 4 °C) and re-suspended in distilled sterile water to attain OD_{600} approximately 2.4 (1.7×10^{10} CFU/ml). To determine cell density in a sample, plate count method was used: cells were serially diluted with distilled water, and then 100 µl of the dilution was plated into Luria broth agar medium. Plates were incubated at 37 °C for 24 h in the incubator, and bacterial colonies were counted manually.

Protein Extraction and Bacterial Inactivation by Means of Electroporation

Overnight culture of *E. coli* cells suspended in distilled water at OD_{600} approximately 2.4 (1.7×10^{10} CFU/ml) were exposed to electric pulses using square wave electric pulse generator HVP-VG (IGEA s.r.l., Carpi, Modena, Italy) and stainless steel plate electrodes, rectangle shape (size of electrode area 0.6 × 2.8 cm) with gap 1 mm. The volume of *E. coli* suspension placed between the electrodes ($d = 1$ mm) was 150 µl. We repeated pulse treatment (each time with new sample) for 10 times in order to get sample volume large enough (approx. 1 ml) for further analysis (determining protein concentration and bacterial inactivation). Sometimes arcing was present when pulses with higher frequencies were applied (8 × 100 µs, 20 kV/cm, 1 kHz). Samples, where arcing occurred, were discarded (not used for further analysis). Different electric pulse protocols were used, where number (8 pulses vs. 32 pulses), duration (100 µs vs. 1 ms), electric field strength (5 vs. 10 kV/cm or 10 vs. 20 kV/cm), and pulse repetition frequency (1 Hz vs. 1 kHz) were varied. In Table 1, all pulse protocols are shown. All experiments were performed at a room temperature (22 °C), where applied electric field (E) was estimated as follows:

$$E = \frac{U}{d}, \qquad (1)$$

where U denotes applied voltage and d electrode distance ($d = 1$ mm). The energy input delivered is reported in Table 1 and was calculated as

Table 1 Set of electric pulse parameters applied to *E. coli* cells with energy input delivered for each condition and the difference in the conductivity of the suspension between first and last pulse applied

Electric pulse parameter	W [J/ml]	R [μS/cm]
(1) 8 × 100 μs; 1000 V (10 kV/cm); 1 Hz	20.80	12
(2) 8 × 100 μs; 2000 V (20 kV/cm); 1 Hz	90.10	58
(3) 8 × 100 μs; 1000 V (10 kV/cm); 1 kHz	23.47	38
(4) 8 × 100 μs; 2000 V (20 kV/cm); 1 kHz	129.28	238
(5) 32 × 100 μs; 1000 V (10 kV/cm); 1 Hz	96.00	51
(6) 32 × 100 μs; 2000 V (20 kV/cm); 1 Hz	533.76	252
(7) 8 × 1 ms; 500 V (5 kV/cm); 1 Hz	5.52	25
(8) 8 × 1 ms; 1000 V (10 kV/cm); 1 Hz	31.68	219

$$W = U \cdot I \cdot \frac{n \cdot T}{V}, \tag{2}$$

where U denotes applied voltage, I current, n number of applied pulses, T pulse duration, and V sample volume (0.15 ml). The conductivity in Table 1 was calculated from the current measured at first and last pulse applied:

$$R = \frac{d}{\delta \cdot A}, \tag{3}$$

where d denotes electrode distance, δ resistance, and A surface of the electrodes.

Protein Extraction and Bacterial Inactivation by Glass Bead Homogenization

Overnight culture of *E. coli* cells suspended in distilled water at OD_{600} approximately 2.4 (1.7×10^{10} CFU/ml) were mixed with glass beads (glass bead diameter was 0.1 mm) at approximate ratio 1:1. Cells were homogenized for 5 min at 2680 rpm with cell disruptor (Disruptor Genie, Carl Roth GMBH, Karlsruhe, Germany). The sample was cooled on ice in order to prevent protein heat disruption.

Analysis of Extracted Protein Concentration and Bacterial Inactivation

After electric pulse application and glass bead homogenization, 50 μl of sample was taken in order to determine the influence of electric pulses and homogenization on *E. coli* viability. Bacterial inactivation was determined with plate count method (Reasoner 2004). Briefly, cells were serially diluted with distilled water and 100 μl of dilution was plated onto Luria broth agar medium. Plates were incubated for 24 h at 37 °C and counted manually. The viability was expressed as log (N/N_0), where N represents the number of colony forming units per ml in treated sample (bacterial cells exposed to electric pulses) and N_0 the number of colony forming units per ml in untreated sample (bacterial cells not exposed to electric pulses).

The rest of the treated sample was filtered through a 0.22 μm filter (Millex-GV; Millipore Corporation, Billerica, MA, USA), and protein concentration was measured by Bradford's assay (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) (Bradford 1976), with bovine serum albumin (BSA) as the standard. Protein concentration was measured also in a sample not exposed to electric pulses or homogenization (untreated sample). Concentration of extracted proteins was obtained as a subtraction of protein concentration in treated sample from protein concentration in an untreated sample.

Statistical Analysis

All experiments were repeated on three different days to check for reproducibility. Results were analyzed using an unpaired t test analysis (SigmaPlot 11.0, Systat Software, Richmond, CA) and were considered statistically different at $P < 0.05$. Each data point in results is the mean value from all three experiments, with standard deviations shown by error bars.

Results

In our study, we focused on relation between extraction of proteins from *E. coli* and bacterial inactivation by means of electroporation. Different electric pulse protocols were tested, where pulse number, duration, and pulse repetition frequency were varied (see Table 1). Bacterial cells were harvested by centrifugation and re-suspended in DH_2O. Afterward, different pulse protocols (Table 1) were used to extract proteins from cells. In parallel, bacterial inactivation was determined. The protein extraction by means of electroporation efficiency was compared also with routine method for protein extraction (glass bead homogenization).

In Fig. 1, the influence of electric field strength is shown in extracted protein concentration and inactivation of *E. coli* bacteria. For all parameters, the increase of electric field strength (from 10 to 20 kV/cm or from 5 to 10 kV/cm) results in the increase of extracted protein concentration ($P < 0.05$) by 2 to 5—times, and in the decrease of bacterial viability ($P < 0.05$) by 2 to 3.4—log reduction.

In Fig. 2, the influence of pulse duration is shown in extracted protein concentration and inactivation of *E. coli* bacteria. Each time, eight pulses were applied with $E = 10$ kV/cm and repetition frequency of 1 Hz. By increasing pulse duration also the protein concentration increases with statistically significant difference ($P = 0.006$), while the decrease in bacterial viability is not

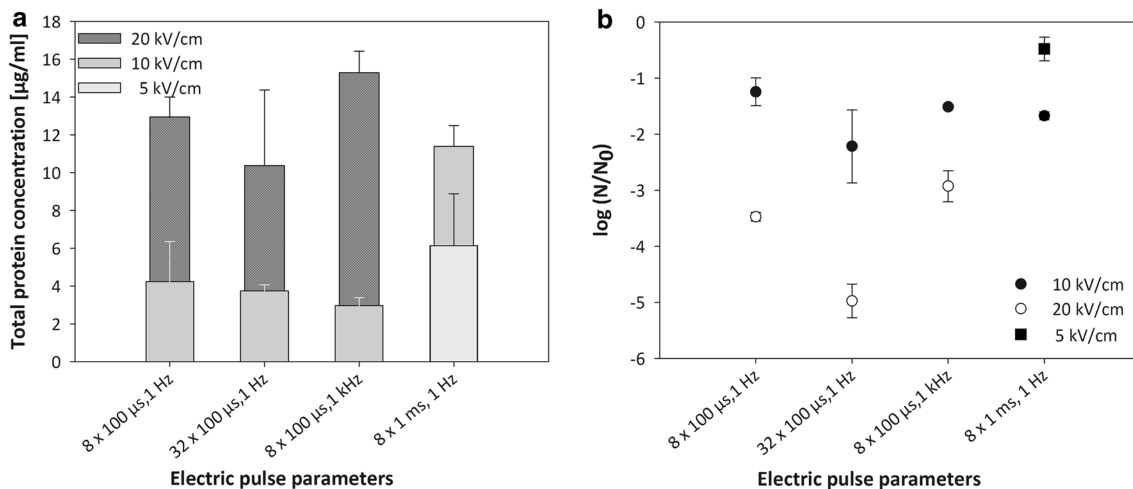

Fig. 1 Effect of electric field strength on **a** extracted proteins and **b** inactivation of *E. coli* bacteria by means of electroporation. Pulses were applied at room temperature (22 °C). Values represent mean ± standard deviation

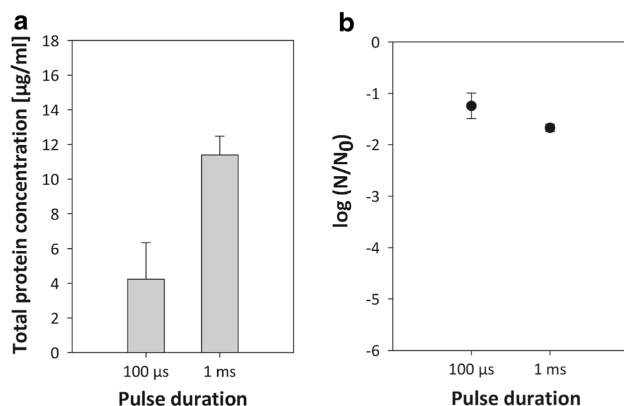

Fig. 2 Effect of pulse duration on **a** extracted proteins and **b** inactivation of *E. coli* bacteria by means of electroporation. Eight pulses with 10 kV/cm of electric field strength and 1 Hz of repetition frequency were applied at room temperature (22 °C). Values represent mean ± standard deviation

statistically significant ($P > 0.05$) and it is 1.25 (100 µs pulses) and 1.67 (1 ms pulses) of log reduction.

In Fig. 3, the effect of pulse number (with pulse duration of 100 µs and pulse repetition frequency of 1 Hz) on protein concentration and inactivation of *E. coli* bacteria is shown. There is no statistically significant difference ($P > 0.05$) in the concentration of extracted proteins for both electric field strengths (10 and 20 kV/cm). Pulse number at lower electric field strength (10 kV/cm) did not affect bacterial viability in a statistically significant manner ($P > 0.05$), while at higher electric field strength (20 kV/cm) bacterial inactivation was influenced by pulse number ($P = 0.007$).

In Fig. 4 the effect of pulse repetition frequency (eight pulses of 100 µs duration) on extracted protein concentration and inactivation of *E. coli* bacteria is shown. When pulse repetition frequency was increased from 1 Hz to 1 kHz, there was no statistically significant difference ($P > 0.05$) neither in the concentration of extracted proteins nor in bacterial inactivation for both electric field strengths (10 and 20 kV/cm).

In order to compare the efficiency of protein extraction by means of electroporation with routine method, we also homogenized bacteria cells with glass beads. We obtained 20.29 µg/ml of proteins with 4.710 of log reduction.

Discussion and Conclusions

Producing valuable proteins in different microorganisms has expanded the area of potential applications. However, methods used to disrupt a biological cell in order to release its intracellular products are all based on total cell disintegration, necessitating further purification steps in order to obtain a pure end-product (Meacle et al. 2004; Salazar and Asenjo 2007). Moreover, chemicals are used in the process, which increases the volume of the sample and represents burden for the environment (Naglak et al. 1990). On the contrary, with application of electric pulses as demonstrated in this study, a quick and chemical-free release of intracellular components from *E. coli* cells (extraction by means of electroporation) is achieved (Ohshima et al. 2000; Shiina et al. 2007; Haberl et al. 2013a; Matos et al. 2013). To improve bacterial viability during extraction by means of electroporation, while still extracting targeted molecule (proteins), pulsing protocol has to be adjusted. Namely, high electric field leads to bacterial death and as a consequence unwanted bacterial membrane toxins (endotoxins) can be released into the sample (Toepfl et al. 2007; Zgalin et al. 2012). Namely, endotoxins have long been

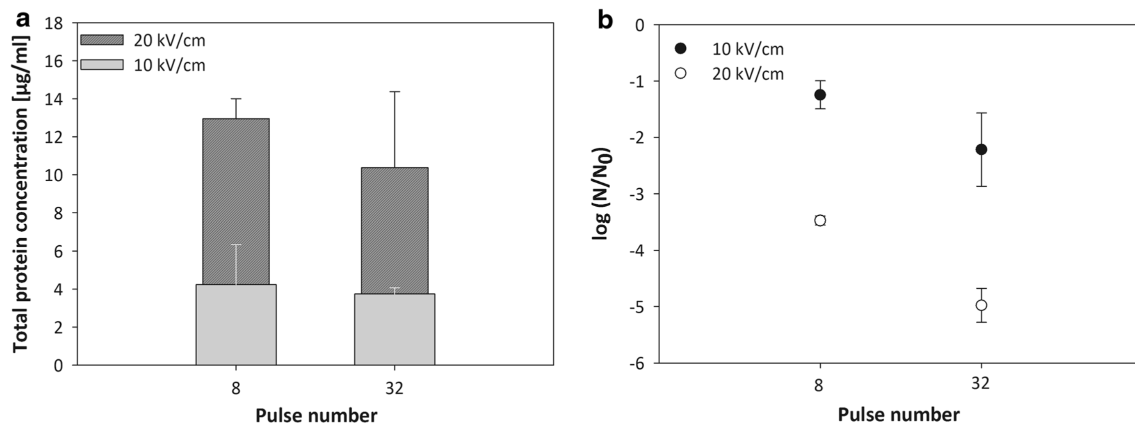

Fig. 3 Effect of pulse number on **a** extracted proteins and **b** inactivation of *E. coli* bacteria by means of electroporation. Pulses of 100 µs duration and 1 Hz of repetition frequency were applied at room temperature (22 °C). Values represent mean ± standard deviation

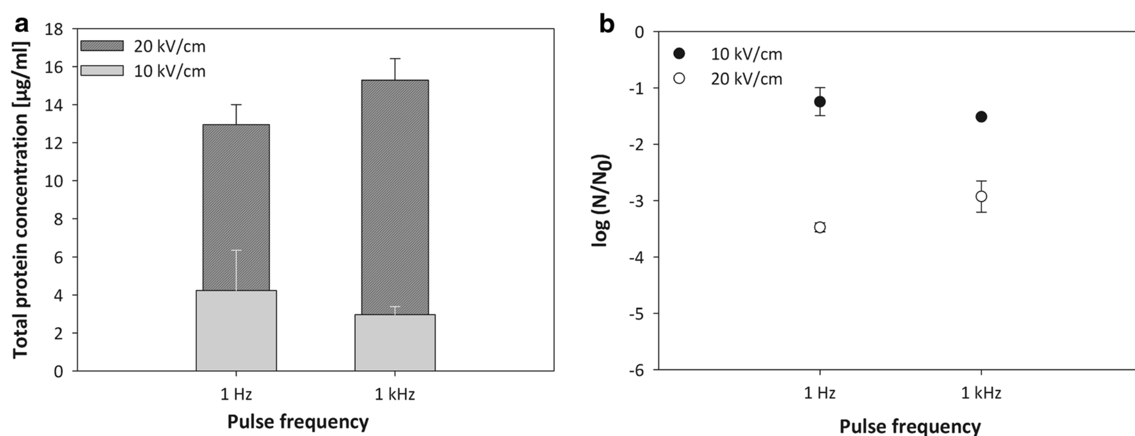

Fig. 4 Effect of pulse repetition frequency on **a** extracted proteins and **b** inactivation of *E. coli* bacteria by means of electroporation. Eight pulses of 100 µs duration were applied at room temperature (22 °C). Values represent mean ± standard deviation

recognized as a key factor in septic shock and are inducing a strong immune response in mammalian cells. Thus, we studied the influence of electric pulse parameters on extracted protein concentration and on bacterial viability. The influence of pulse strength, duration, number, and repetition frequency was analyzed (see Table 1).

Electric pulse parameters for the extraction purposes vary greatly on selected organism. Namely, mammalian, yeast, and microalgae cells are larger than bacterial cells; therefore, lower field intensities are needed in order to permeabilize the membrane and extract proteins from the cells. Therefore, comparison of our results with similar research on other cells must be done with caution (Suga et al. 2007; Suga and Hatakeyama 2009; Zhan et al. 2010, 2012; Coustets et al. 2013).

Furthermore, in most of the studies, authors focused on maximum protein extraction with no concern to cell viability. Ohshima et al. showed that with 10 kV/cm of electric field strength, 1 µs pulse duration and frequencies up to 50 Hz intracellular protein were extracted with nine times higher specific activity compared to ultrasound method (Ohshima et al. 2000). Although the effectiveness cannot be attributed only to electric pulse parameters, polyethylene glycol was added, which increases osmotic pressure. In our case, however, no chemical agents were added (bacterial cells were suspended only in distilled water); therefore, the only parameter that influences protein extraction would be the electroporation.

Our results show that by increasing electric field strength, the concentration of extracted proteins increases and viability decreases (see Fig. 1). The relation seems reasonable, since higher electrical field destroys bacterial envelope, releasing all intracellular components into surrounding media (Garcia et al. 2007; Saulis 2010; Zgalin et al. 2012). Although high electric field yields maximum amount of extracted proteins, it is not the best choice, since other unwanted molecules could be present (e.g., endotoxins), making downstream process more complicated and expensive. Therefore, our aim was to achieve high bacterial viability and to gain as much proteins as possible. Although

we used one of the most frequently used protocol (filtration) to separate cells from extracted proteins, some proteins were still trapped on the filter. Therefore, in our case, the yield of extracted proteins by means of electroporation is even higher than reported.

Another electric parameter to be considered is pulse duration. Namely, it has been shown in mammalian (Wolf et al. 1994; Rols and Teissie 1998; Haberl et al. 2013b) and bacterial cells (Xie and Tsong 1992; Garcia et al. 2007; Coustets et al. 2015) that pulse duration largely affects permeabilization of the cell membrane as well as cell viability. Xie et al. showed that pulses of more than 1 ms duration decrease *E. coli* viability (Xie and Tsong 1992). However, in our experiments, maximum pulse duration was 1 ms; thus, it seems like that pulse durations up to 1 ms at lower amplitudes (10 kV/cm) does not affect bacterial viability (see Fig. 2b. This could also be attributed to small difference in energy input for both pulse durations (in 100 µs pulses $W = 20.80$ J/ml and in 1 ms pulses $W = 31.68$ J/ml). Under our experimental conditions (8 pulses at 10 kV/cm), bacterial membrane seems to be reversibly permeabilized; therefore, no significant effect of pulse duration was observed on bacterial viability. Our experiments also suggest that by increasing pulse duration, membrane permeabilization increases, since more extracted proteins were obtained at 1 ms pulses (see Fig. 2a). Higher protein concentration obtained at longer pulses could also be attributed to the electrophoretic force, which could drag charged proteins from the permeabilized bacterial membrane. Namely, proteins could be stumbled in membrane pores and longer pulses could drag charged proteins from the bacteria. But this theory needs to be thoroughly studied.

The number of pulses (see Fig. 3) in our case has no effect on the concentration of extracted proteins, while the effect on bacterial viability was electric field strength dependent. Meaning that at higher voltages (20 kV/cm) bacterial viability dropped by 1.5 log when pulse number was increased (from 8 to 32 pulses). The same effect was observed also in previous study on *E. coli* cells, where the number of pulses did not have a significant effect on survival at lower voltages, whereas at higher voltages minor impact was observed (Xie and Tsong 1992). The influence of pulse number on bacterial viability is also bacteria strain depended. Namely, no notable effect was observed on inactivation of gram-positive bacteria, *Bacillus cereus*, when pulse number was increased (Bermudez-Aguirre et al. 2012). Gram-positive bacteria have much thicker peptidoglycan layer, which seems to be more electric pulse resistant.

The amount of extracted proteins is independent of pulse repetition frequency at our values (1 Hz and 1 kHz) (see Fig. 4), while bacterial viability was affected only at higher electric field strength (20 kV/cm). As it was shown by Asavasanti et al., low pulse frequencies (below 1 Hz) yield a higher degree of plant tissue permeabilization than higher pulse frequencies (above 1 Hz) (Asavasanti et al. 2011). In a similar way, for our conditions, frequencies lower than 1 Hz could yield higher protein extraction, but this needs to be tested.

Bacterial cells were subjected to electric pulses in the stationary phase. According to the literature, bacterial membrane is most susceptible to electric pulse permeabilization at middle or late exponential phase (Coustets et al. 2015). But we did not observe any difference in protein extraction (or bacterial viability) for bacteria subjected to electric pulses in early or late exponential phase growth phase (data not shown).

In general, higher protein extraction is associated with lower bacterial viability and vice versa (Fig. 5), as bacterial viability has negative correlation coefficient with extracted protein concentration ($r = -0.55$).

Higher concentration of proteins can be extracted with higher electric field strength, but at those conditions bacterial viability is largely affected. Therefore, the most appropriate parameters to be used for protein extraction with minimal effect on bacterial viability would be parameters above the regression line (pulse parameters 1, 4, 7, and 8—see Fig. 5; Table 1). Thus, preferred are lower pulse amplitudes of longer pulse duration, where bacterial membrane is reversibly electroporated and the bacterial cell survives. Based on our experiments, the most promising pulse protocol for protein extraction by means of electroporation are longer pulses (1 ms) with lower pulse amplitude (up to 5 kV/cm). At those conditions, reversible pores seemed to form in bacterial membrane, releasing intracellular proteins while cell was still alive. Our results

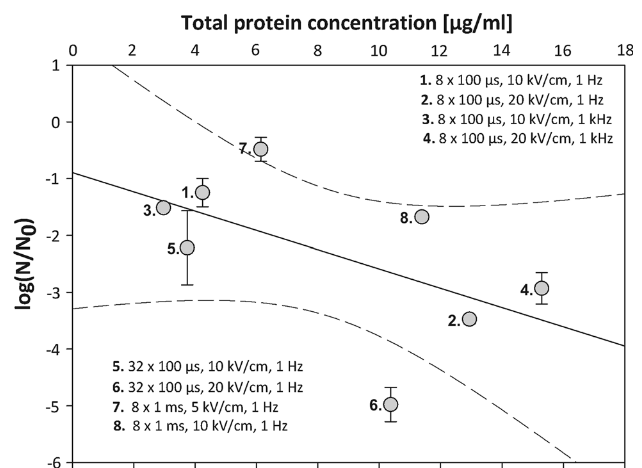

Fig. 5 The correlation between the concentration of extracted proteins and bacterial viability. Linear regression line suggests negative dependence of the variables ($r = -0.55$)

are consistent with study of Garcia et al., where they showed that increased electric field and/or treatment time (pulse duration multiplied with pulse number) reduces the bacterial viability and proportionally increases permeabilization of cell membrane (Garcia et al. 2007). Although extraction of proteins by means of electroporation is currently less effective than glass bead homogenization (according to our results), it has several advantages, such as high speed of extraction, less contaminants (the cost of protein purification at large scales should be lower), since bacterial cells are not totally disintegrated as shown by scanning electron microscope (see Fig. 6).

Namely, when bacterial cells were subjected to pulses, where the lowest viability was observed (32 × 100 µs, 20 kV/cm, 1 Hz), no bacterial disintegration occurred (see Fig. 6c d). In gram-negative bacteria (as it is *E. coli*), cell wall is covered with outer membrane (on SEM pictures outer membrane looks like curly envelope). After electroporation, we did not observe bacteria disintegration. Therefore, we cannot say that cell wall destruction plays a role in the inactivation of cells by electroporation or extensive membrane damage is a key event in the bacterial inactivation.

But when bacterial cells were mechanically disrupted with glass beads, cells were totally broken down (see Fig. 6e, f), and simple filtration could not separate the proteins from other cell debris. Also other unwanted molecules (e.g., endotoxins) may be present in the final sample. In order to confirm that, of course, other methods should be used (LAL test to detect endotoxins).

In order to evaluate the effect of the energy input delivered in each condition (see Table 1) on protein

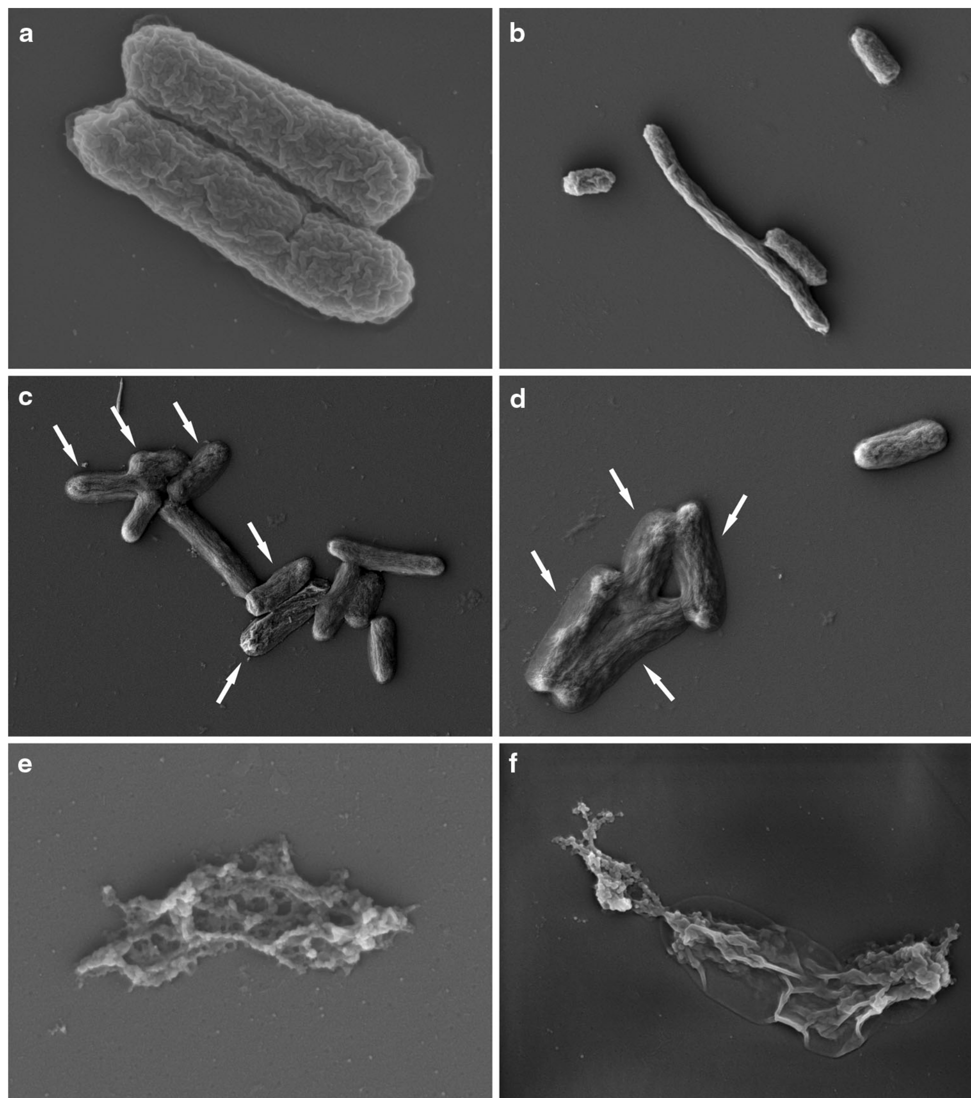

Fig. 6 SEM images of bacterial cells in **a** and **b** control sample; **c** and **d** sample subjected to electric pulses (32 × 100 µs, 20 kV/cm, 1 Hz). White arrows indicate non-viable cells; **e** and **f** sample subjected to glass bead homogenization

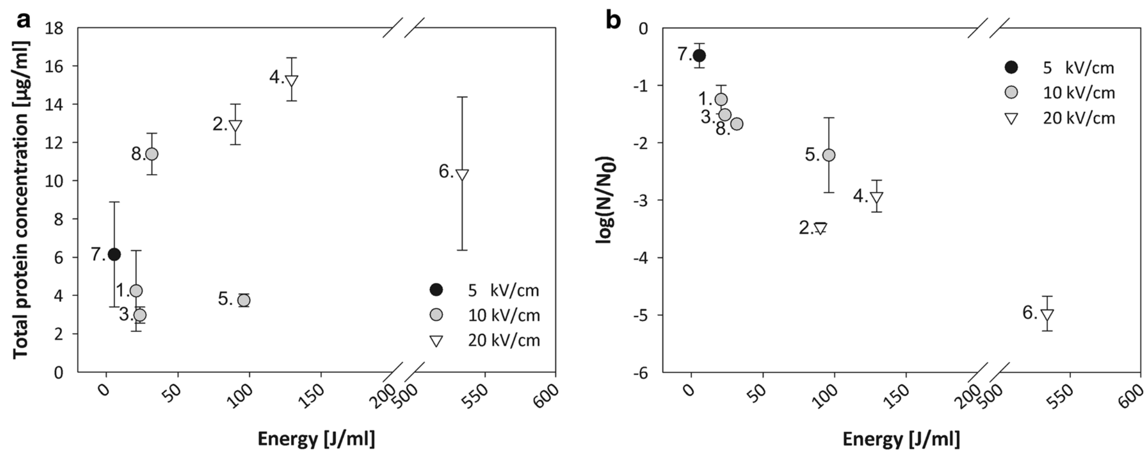

Fig. 7 The effect of the energy input delivered in each condition on **a** extracted proteins and **b** inactivation of *E. coli* bacteria by means of electroporation. Pulses were applied at room temperature (22 °C). Electric pulse parameters were as follows: (1) 8 × 100 µs, 10 kV/cm, 1 Hz; (2) 8 × 100 µs, 20 kV/cm, 1 Hz; (3) 8 × 100 µs, 10 kV/cm, 1 kHz; (4) 8 × 100 µs, 20 kV/cm, 1 kHz; (5) 32 × 100 µs, 10 kV/cm, 1 Hz; (6) 32 × 100 µs, 20 kV/cm, 1 Hz; (7) 8 × 1 ms, 5 kV/cm, 1 Hz; (8) 8 × 1 ms, 10 kV/cm, 1 Hz. Values represent mean ± standard deviation

extraction and bacterial viability (see Fig. 7), we used as processing variables the field strength and the total specific energy input, being the latter an integrated parameter which accounts for the number of pulses and the pulse width.

The energy input seems not to directly correlate with extracted proteins. Namely, at the highest energy input (and at the highest electric field strength-20 kV/cm), we did not obtain also the highest proteins concentration (see Fig. 7a, parameter 6.). The same was observed also when applied electric field strength was 10 kV/cm. Also at the lowest energy input (5.52 J/ml) and lowest electric field strength (5 kV/cm), the concentration of extracted proteins was higher than, i.e., at electric field strength 10 kV/cm and energy inputs 20.80, 23.47, and 96 J/ml. The same observations were made by Ohshima et al., where the specific energy input did not affect total protein extraction (Ohshima et al. 2000). Nevertheless, the effect of delivered energy and electric field strength on bacterial viability is coherent. Bacterial viability increases with both more intense applied electric field strength (20 kV/cm) and higher total specific energy input (533.76 J/ml) delivered to the *E. coli* suspension, which is in agreement with other studies (Pataro et al. 2010, 2011).

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Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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