Bioelectrochemistry 112 (2016) 77–82

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Short communication

The effect of temperature and bacterial growth phase on protein extraction by means of electroporation

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ARTICLE INFO

Article history:
Received 30 December 2015
Received in revised form 1 August 2016
Accepted 15 August 2016
Available online 16 August 2016

Keywords:
Electroporation
Escherichia coli
Bacterial inactivation
Temperature
Bacterial growth phase

ABSTRACT

Different chemical and physical methods are used for extraction of proteins from bacteria, which are used in variety of fields. But on a large scale, many methods have severe drawbacks. Recently, extraction by means of electroporation showed a great potential to quickly obtain proteins from bacteria. Since many parameters are affecting the yield of extracted proteins, our aim was to investigate the effect of temperature and bacterial growth phase on the yield of extracted proteins. At the same time bacterial viability was tested. Our results showed that the temperature has a great effect on protein extraction, the best temperature post treatment being 4 °C. No effect on bacterial viability was observed for all temperatures tested. Also bacterial growth phase did not affect the yield of extracted proteins or bacterial viability. Nevertheless, further experiments may need to be performed to confirm this observation, since only one incubation temperature (4 °C) and one incubation time before and after electroporation (0.5 and 1 h) were tested for bacterial growth phase. Based on our results we conclude that temperature is a key element for bacterial membrane to stay in a permeabilized state, so more proteins flow out of bacteria into surrounding media.

1. Introduction

Genetic engineering has opened a possibility to produce proteins for medicine and industry in recombinant bacteria [1]. The growing relevance of this field is highlighted by the fact that demand for biological molecules is increasing rapidly [2]. 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 production costs are lower [3–6]. 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) [7], in biochemistry applications (horseradish peroxidase used to amplify a weak signal of a target molecule) [8], *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 [9]. 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 [2]. In order to extract recombinant proteins from bacterial cells, various methods have been used – chemical and physical ones [10]. Main drawbacks of chemical methods used for obtaining recombinant proteins from bacteria are: (i) use of expensive chemicals, which are often also toxic and are on pharmaceutical production scale restricted by regulatory bodies; (ii) different bacteria sensitivity towards various chemicals; (iii) high cost; or (iv) are time consuming. While physical methods are effective for different bacteria species, they still have certain disadvantages: (i) extensive bacteria fragmentation and/or protein denaturation; (ii) non-selective extraction of proteins; (iii) high heating; or (iv) difficulties in handling large volumes [10]. In order to overcome these shortfalls new extraction methods have to be developed. One of the promising methods for extracting intracellular products from cells was found to be electroporation [11].

Namely, when a cell membrane is subjected to electric pulses of adequate strength and induced transmembrane voltage surpasses a certain value, the cell membrane becomes transiently permeable [12]. Thus small or large molecules can be introduced into or extracted from cells. Electroporation is now used in different fields: clinics (electrochemotherapy, gene electrotransfer, irreversible tissue ablation, DNA vaccination) [13–16]; food industry (inactivation of microorganisms, drying, extraction of juice from fruits and vegetables) [17–19]; and biotechnology (bacterial electrotransformation, extraction of technologically relevant molecules from microorganisms) [20–23].

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http://dx.doi.org/10.1016/j.bioelechem.2016.08.002
1567-5394/

In studies reported hitherto researches have shown that extraction of proteins by means of electroporation is non-selective and can be used to obtain proteins from various (micro)organisms, such as microalgae [24], yeast [11], bacteria [25] or eukaryotic cells [26]. The efficiency of method was found to be strongly dependent on electric pulse parameters. Ohshima et al. showed that the amount of proteins obtained from yeast cells is increasing with electric field strength [11]. Although the maximum amount of extracted proteins was only 30% of the amount obtained with glass bead homogenization, electroporation has a great advantage of allowing much faster protein extraction. The influence of electric pulse parameters on protein extraction also from bacterial cells was studied previously [21]. Main conclusions in this study were, that pulse parameters need to be carefully selected in order to extract proteins, but at the same time to prevent extensive bacterial disintegration. Recently, it was reported that millisecond duration pulses can be used for extracting proteins from *E. coli* cells in a pre-industrial pilot flow-through system. Authors observed that the yield of extracted proteins was strain, bacterial growth phase, pulse condition and temperature dependent and that a right balance between these parameters is needed [25].

Therefore our aim was to study the effect of temperature on extraction of proteins by means of electroporation from bacterial cells. *E. coli* cells were incubated at different temperature prior and after electroporation. Furthermore, since bacterial growth phase strongly affects cell wall porosity and was shown to influence the efficiency of protein extraction [25], we also studied the effect of bacterial growth phase (early exponential, middle exponential and stationary phase) on protein extraction. At the same time in addition to protein extraction we also determined bacterial viability.

2. Material and methods

2.1. Preparation of bacterial cells

In our study we used *Escherichia coli* K12 TOP10 strain carrying plasmid pEGFP-N1 (Clontech Laboratories Inc., Mountain View, CA, USA), which encodes kanamycin resistance. Bacterial cells were inoculated in Luria Broth medium (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) with 50 μg/ml of antibiotic kanamycin sulphate (Carl ROTH Gmbh, Essen, Germany). After agitation at 37 °C, cell pellet was collected by centrifugation (4248 ×*g*, 30 min, 4 °C) and re-suspended in distilled water to attain 1.6×10^9 CFU/ml. Cell density was determined by plate count method, where bacterial cells were serially diluted with distilled water, and then 100 μl of the dilution was plated into Luria broth agar medium (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Plates with inoculated bacteria were incubated at 37 °C for 24 h in the incubator, and bacterial colonies were counted manually.

2.1.1. The temperature effect on protein extraction by means of electroporation

After 17 h agitation at 37 °C, bacterial cells were incubated at different temperatures (see Table 1) for 0.5 h and exposed to electric pulses. After the exposure of cells to electric pulses bacterial cells were incubated for 1 h at various temperatures (see Table 1) and then analysis was made (see Section 2.3).

Table 1
Incubation temperatures of *E. coli* cells before and after electroporation.

Before electroporation (for 0.5 h)	After electroporation (for 1 h)
4 °C	4 °C
22 °C	22 °C
37 °C	37 °C
45 °C	45 °C
4 °C	37 °C
37 °C	4 °C

2.1.2. The bacterial growth phase effect on protein extraction by means of electroporation

After 6, 11 or 17 h agitation at 37 °C, bacterial cells were incubated at 4 °C for 0.5 h and exposed to electric pulses. Following this treatment, bacterial cells were again incubated for 1 h at 4 °C and then analysis was made (see Section 2.3).

2.2. Extraction of proteins by means of electroporation

After 0.5 h incubation at different temperatures (4, 22, 37 or 45 °C) *E. coli* cells (150 μl) were placed between stainless steel plate electrodes, rectangle shape (size of electrode area 0.6 × 2.8 cm) with distance 1 mm between the plates and exposed to electric pulses at room temperature using square wave electric pulse generator HVP-VG (IGEA s.r.l., Carpi, Modena, Italy). Pulse treatment was repeated 11-times (each time with new sample) in order to obtain sufficiently large volume for further analysis. All samples were immediately after electroporation collected in a tube, which was held at temperature, specified in Table 1.

A train of eight pulses with 1 ms duration, 5 kV/cm of electric field strength and 1 Hz of pulse repetition period were applied. The electric field (E) was estimated as:

$$E = \frac{U}{d} \tag{1}$$

where U represents applied voltage and d electrode distance (d = 1 mm). Although at higher electric fields more proteins can be extracted, we chose lower electric field (5 kV/cm), where no arcing is present. Namely, arcing is detrimental for pulse generator, furthermore it leads to inhomogeneous electric field distribution, ionization and shock wave generation, so treated samples where arcing occurs are not comparable with those where arcing was not present.

Bacterial cells in control were handled in all aspects equally but no electric pulses were delivered. The conductivity of bacterial suspension was measured by conductivity meter (Mettler-Toledo International Inc.), and was 171.3 μS/cm.

2.3. Electropermeabilization

To evaluate electropermeabilization of bacterial cells propidium iodide (PI) was used. PI is a molecule which enters cell, if its membrane is permeabilized [27]. Bacterial cells were prepared as described in Section 2.1. Immediately before electric pulse application PI was added (final concentration of PI in a sample was 100 μg/ml) and 400 μl of bacterial suspension was placed in a cuvette with built in aluminum electrodes (d = 2 mm). Samples were then exposed to electric pulses to deliver PI into the cells using square wave prototype pulse generator [28]. Electric parameters were the same as described in Section 2.2. After pulses were applied, bacterial cells were incubated for 15 min in the dark at room temperature (22 °C) and then centrifuged for 4 min at 12,000 ×*g* at 22 °C to remove extracellular PI that did not enter the cells. Pellet was re-suspended with 400 μl of distilled water and the uptake of PI was evaluated with spectrofluorometer (Tecan infinite M200, Tecan Austria GmbH) at 617 nm.

The permeabilization (uptake of PI) was defined as:

$$Permeabilization\ (\%) = \frac{FL(E) - FL(E=0)}{FL(max) - FL(E=0)} \tag{2}$$

where $FL(E)$ denotes fluorescence intensity of cells subjected to electric pulses, $FL(E=0)$ fluorescence intensity of cells at $E = 0$, *i.e.* cells in control, and $FL(max)$ maximum fluorescence intensity, *i.e.* where saturation fluorescence is achieved. For obtaining maximum cell disruption we incubated bacterial cells with 1% Triton detergent for 1 h.

2.4. Determining total cell protein content with glass bead homogenization

E. coli cells were re-suspended in distilled water to attain 1.6×10^9 CFU/ml and then 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).

2.5. Determination of extracted proteins and bacterial viability

After exposure of cells to electric pulses and 1 h incubation at given temperature, 50 μl of *E. coli* cells were taken from the sample, diluted and plated into Luria broth agar medium. Bacterial viability was determined with plate count method [29]. Plates with inoculated bacteria were incubated for 24 h at 37 °C and counted manually. The viability was presented as log (N/N_0), where N represents the number of CFU/ml in sample exposed to electric pulses and N_0 the number of CFU/ml in control sample (*E. coli* cells not exposed to electric pulses, otherwise treated in identical manner).

The rest of the sample was filtered through a 0.22 μm filter (Millex-GV; Millipore Corporation, Billerica, MA, USA). Protein concentration was determined with Bradford's assay (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) [30], with bovine serum albumin (BSA) as the standard. Concentration of extracted proteins - *c* (*extracted*) - was determined as:

$$c\,(extracted) = c\,(pulses) - c\,(control) \quad (3)$$

where *c* (*pulses*) represents a protein concentration in a sample exposed to electric pulses and *c* (*control*) represents a protein concentration in a sample not exposed to electric pulses, but otherwise treated in identical manner as treated sample.

2.6. Statistical analysis

Experiments were repeated two or three times, on three different days to prove repeatability. Results were evaluated using an unpaired *t*-test analysis (SigmaPlot 11.0, Systat Software, Richmond, CA) and were considered statistically different at $P < 0.05$. Each bar or data point in results represents the mean value from two or three experiments, with standard deviations shown as error bars.

3. Results and discussion

By electrically inducing changes in bacterial membrane, leakage of intracellular components can occur. The method is termed extraction by means of electroporation (also electroextraction) and various parameters are affecting its efficiency (electric pulse parameters, temperature, growth phase of bacterial cells *etc.*). In order to increase the efficiency and to avoid the presence of bacterial endotoxins, which can be released from damaged bacterial membrane in non-viable cells, optimization of electric pulse parameters is needed. Namely endotoxins can elicit immune response in mammals and are therefore unwanted molecules in final sample. Thus we also checked in parallel with protein extraction bacterial viability for all incubation temperatures and bacterial growth phases.

For our experiments we used distilled water as a medium in which bacterial cells were diluted and electroporated. We are aware that in distilled water the protein native conformation is changed, but since we are limited by the capacity of our pulse generator, we electroporated bacteria in low conductivity medium (*e.g.* distilled water). Namely, with higher conductivity of the sample also strong arcing is present (with excessively high currents-more than 100 A was measured), which makes samples incomparable to those where arcing was not present. Furthermore, in our study the extraction was also selective, as we extracted only water soluble proteins, whereas other proteins remained inside the cell.

In order to compare our results with other extraction techniques, we estimated the protein yield as [g proteins/kg dry weight], where dry weight of one *E. coli* bacteria was obtained from the literature [31].

Also the percentage of permeabilization for PI, which represents the uptake of PI was evaluated.

3.1. The temperature effect on protein extraction by means of electroporation

Temperature has a significant effect on cell membrane structure and by that on permeabilization of the cell membrane. It was also shown that post-pulse incubation at lower temperatures could promote leakage of proteins from bacterial cells [31], since its membrane does not reseal as quickly as it would at higher temperatures (*e.g.* at room temperature or at 37 °C). Furthermore, it was shown on algal cells that electroporation combined with temperature could not sufficiently disrupt algal membrane to extract proteins in a comparable manner as glass bead milling [32]. Therefore our motivation was to observe the effect of different pre- and post-temperatures (see Table 1) on protein extraction and also on bacterial viability after electroporation.

In Fig. 1 the concentration of proteins extracted by means of electroporation as a function of incubation temperature is shown, where bacterial cells were incubated at the same temperatures before and after electroporation. Our results show that pre- and post-incubation at 4 °C increases concentration of extracted proteins by 1.4 –, 1.8 – and 2.4 – times compared to pre- and post-incubation at 22 °C ($P = 0.010$), 37 °C ($P = 0.008$) and 45 °C ($P < 0.001$), respectively. Thus our hypothesis is that if we increase the incubation temperature after electroporation, we decrease the time window for protein leakage from bacterial cells, which is consistent with faster membrane resealing. If we compare our results with glass bead homogenization (36.11 μg/ml of proteins), we extracted approximately 18.8, 11.9, 10.8 and 6.7% of total protein content at 4, 22, 37 and 45 °C, respectively. Meanwhile, it seems like

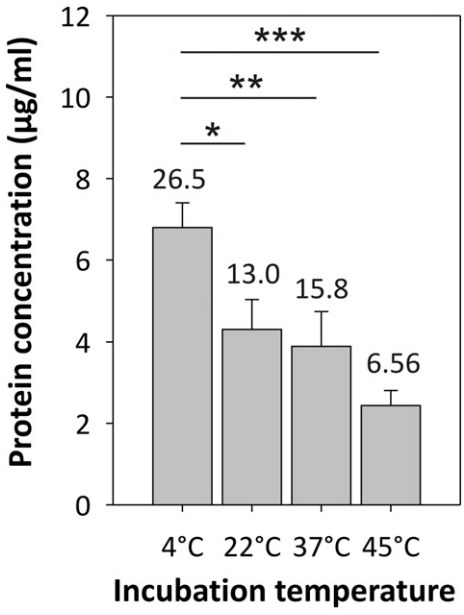

Fig. 1. The effect of incubation temperature on protein extraction by means of electroporation. Before electroporation bacterial cells were incubated at various temperatures (see Table 1) and then exposed to eight pulses with 5 kV/cm of electric field strength, 1 ms pulse duration and 1 Hz of repetition frequency. After pulsing bacterial cells were again incubated at different temperatures (see Table 1). *P* values are: *0.01, **0.008, ***< 0.001. Values represents means ± standard deviation, numbers above bars represents the estimation of g proteins/kg dw.

Table 2
The effect of incubation temperature on bacterial viability. Values represents means ± standard deviation.

Incubation temperature before and after the treatment	N_0 (number of CFU colonies in a control sample)	N (number of CFU colonies after the treatment)	Log N/N_0
4 °C	1.28×10^9	4.47×10^8	-0.46 ± 0.33
22 °C	1.66×10^9	1.01×10^9	-0.21 ± 0.30
37 °C	1.23×10^9	5.55×10^8	-0.35 ± 0.17
45 °C	1.86×10^9	7.37×10^8	-0.40 ± 0.04

there was no statistically significant difference in bacterial viability for all incubation temperatures (see Table 2).

Each bacterial species has specific cardinal temperatures. Those are temperatures at which growth rate is at minimum, optimum or maximum. For *E. coli* cells minimum temperature at which membrane gelling and stopping of growth occurs is 8 °C, optimum temperature at which enzymatic reactions are at maximum is 39 °C and maximum temperature at which protein denaturation and collapse of cytoplasmic membrane occurs is found to be 48 °C [33]. Temperatures of course vary between different strains of *E. coli*. We believe that incubating bacterial cells 0.5 h prior electroporation at 4 °C did not cause membrane gelling, but rather caused (after electroporation) a prolonged membrane permeabilization, since more proteins were extracted at 4 °C. In order to confirm our statement, we checked the electropermeabilization at 4, 22, 37 and 45 °C, where 32.8 ± 8.3, 27.2 ± 8.1, 28.7 ± 7.5 and 31.3 ± 11.7% of bacterial cells were permeabilized, respectively. We believe if bacterial membrane would be already in a gelled state, the electropermeabilization at 4 °C would be lower, compared to other temperatures.

Since it was shown on mammalian cells that incubating cells at lower temperatures (4 °C) prior or during electroporation significantly decreases effectiveness of membrane permeabilization [34], our motivation was also to observe if pre-incubation at 37 °C would increase the degree of membrane fluidity and by that the permeabilization, and post-incubation at 4 °C would then prolonged the permeabilization state of the membrane – thus more proteins would be extracted. Furthermore less energy input would be needed to achieve same effect. However, we did not observe any increase neither in the concentration of extracted proteins nor in bacterial viability (compared to pre- and post-incubation at 4 °C). In Fig. 2 the concentration of proteins extracted by means of electroporation as a function of incubation temperature is shown, where bacterial cells were incubated at different temperatures before and after electroporation. When changing the pre- and post-incubation temperature (from 4 °C to 37 °C and *vice versa*), the concentration of extracted proteins did not increase ($P = 0.444$), and represents approximately 13% of total protein content. Also there was no effect on bacterial viability ($P = 0.333$) (Table 3).

Since we did not observe any difference in the yield of extracted proteins by means of electroporation, when changing the pre- and post-incubation temperature (see Fig. 2), we believe that maybe longer incubation times would be needed in order to influence on membrane behavior of bacterial cells and/or detect differences. It also seems like the mechanism of bacterial membrane fluidity can't be correlated to mammalian one.

3.2. The bacterial growth phase effect on protein extraction by means of electroporation

Bacterial growth phase strongly affects bacterial metabolism. In exponential growth phase cells rapidly grow and are metabolically very active. In stationary phase no net increase or decrease in cell number occur, but many cell functions still continue, such as energy metabolism as well as some biosynthetic processes [33]. It was shown that bacterial cells at different growth phases respond differently to electroporation [31]. Therefore, our motivation was to study the effect of bacterial

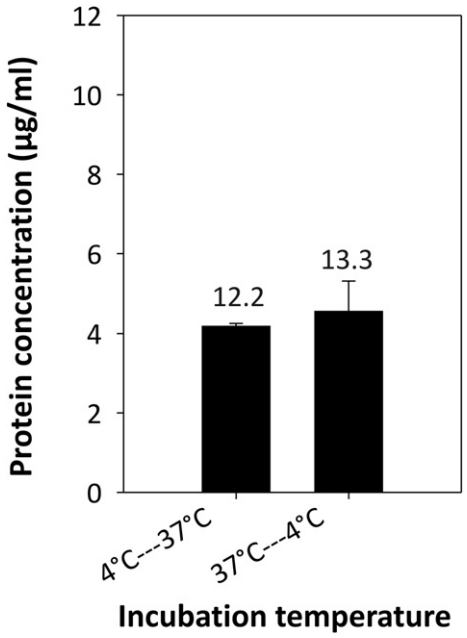

Fig. 2. The effect of incubation temperature on protein extraction by means of electroporation. Before electroporation bacterial cells were incubated at various temperatures (see Table 1) and then exposed to eight pulses with 5 kV/cm of electric field strength, 1 ms pulse duration and 1 Hz of repetition frequency. After pulsing bacterial cells were again incubated at different temperatures (see Table 1). Values represents means ± standard deviation, numbers above bars represents the estimation of g proteins/kg dw.

growth phase on protein extraction. Growth phases after 6, 11 or 17 h were previously determined for our strain (see Appendix 1). In Fig. 3 the concentration of proteins extracted by means of electroporation as a function of bacterial growth time prior electroporation is shown. Bacterial cells were before and after electroporation incubated at 4 °C. We observed no statistically significant effect on extracted proteins or on bacterial viability (Table 4) when bacterial cells were electroporated after 6, 11 or 17 h of growth time. Compared to total protein content, we extracted 15.6, 12.2 and 11.7% of proteins after 6, 11 or 17 h of growth time, respectively.

In order to avoid the influence of different batch cultures, we extracted proteins by means of electroporation from the same batch bacterial culture agitated for 6, 11 or 17 h. Before the experiment we adjusted the bacterial density (diluted bacterial cells in order to obtain the same OD_{600} for all hours of incubation). Thus, approximately the same amount of bacterial cells was exposed to electric pulses after 6, 11 or 17 h of agitation.

During growth of bacteria the ratio of protein to lipid content in bacterial membrane changes. As bacteria enter the stationary growth phase, more proteins are present in its membrane compared to lipids, therefore decreased membrane fluidity and consequently permeabilization of bacteria in stationary phase is expected [35]. Furthermore, also cell wall porosity strongly depends on growth phase of bacteria. Namely, peptidoglycans' (major component of bacterial cell wall)

Table 3
The effect of incubation temperature on bacterial viability. Values represents means ± standard deviation.

Incubation temperature before the treatment	Incubation temperature after the treatment	N_0 (number of CFU colonies in a control sample)	N (number of CFU colonies after the treatment)	Log N/N_0
4 °C	37 °C	1.70×10^9	7.40×10^8	-0.36 ± 0.20
37 °C	4 °C	9.40×10^8	3.51×10^8	-0.43 ± 0.50

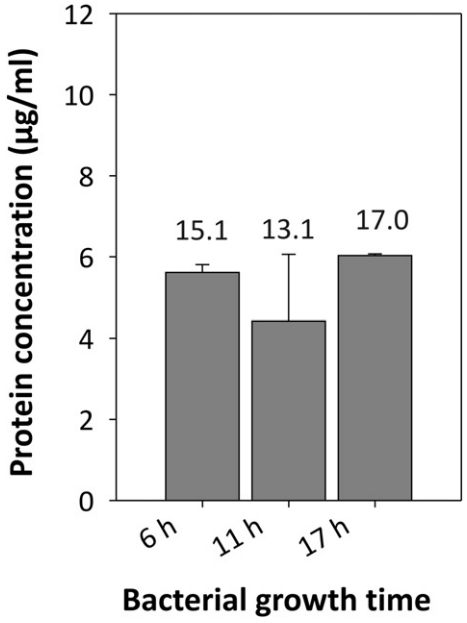

Fig. 3. The effect of bacterial growth time on protein extraction by means of electroporation. Before electroporation bacterial cells were incubated at 4 °C and then exposed to eight pulses with 5 kV/cm of electric field strength, 1 ms pulse duration and 1 Hz of repetition frequency. After pulsing bacterial cells were again incubated at 4 °C. Values represents means ± standard deviation, numbers above bars represents the estimation of g proteins/kg dw.

chemistry is dependent on bacterial growth phase. The levels of penicillin binding protein, which is involved in the final stages of the synthesis of peptidoglycan, diminish very rapidly in the stationary growth phase [36]. It was also previously shown that in stationary phase: (i) inner membrane of Gram negative bacteria (*e.g. E. coli*) is in highly ordered structure, with reduced fluidity, (ii) the peptidoglycan layer (bacterial cell wall component) has increased thickness [37], (iii) bacteria is smaller and in more spherical shape [38] and (iv) cells are making cell clusters, since they are more adhesive due to the high degree of local charge heterogeneity on the outer membrane [39]. All these parameters support the hypothesis that bacteria in a stationary growth phase would be less prone to the electric pulses. However, our results did not support this hypothesis and are not in agreement with other studies [25,31,35,40]. Our bacteria were electroporated at late exponential phase (6 h of growth time) and in stationary phase (11 and 17 h of growth time). It needs to be stressed however that different parameters (temperature, electric pulses) or bacteria were used in those studies and that further experiments need to be done for our strain after 3 h (early exponential phase) or 5 h (middle exponential phase) of growth time. In all listed studies the cells were most susceptible to electric pulses in earlier stages of growth. Electrotransfection efficiency of bacterial cells was the highest when cells were electroporated in middle exponential phase and lowest when cells were in stationary phase [31,40]. In study of Loghavi et al., authors concluded that membrane permeability for dyes is highest in earlier stages of bacterial growth. When extracting proteins by means of electroporation authors observed that in stationary growth phase the lowest amount of proteins was extracted [25]. The discrepancy between the results could be due to the fact, that we used different experimental conditions. We extracted proteins in a batch system (plate stainless steel electrodes); while in a study of Coustets et al. they used a flow electroporation (continuous mode). From their report it is not clear, if they used the same batch bacterial culture in different growth phases. Furthermore, they incubated cells after electroporation for 4 h in a media containing 0.2 M Tris, 3 mM EDTA and 5 mM DTT, which affects bacterial membrane and cell wall. We incubated cells 1 h after electroporation only in distilled water, enabling bacterial membrane to reseal without any other chemicals in media. In other studies transformation efficiency was observed [31,40], and we believe that the mechanism of bacterial transformation is different from that of extraction, where membrane needs to stay in a electroporated state (*i.e.* of increased permeability state) as long as possible in order for proteins to be extracted from cells into surrounding media. Finally, the susceptibility of bacteria to electric pulses could also be bacterial strain depended.

Table 4
The effect of bacterial growth time on bacterial viability. Values represents means ± standard deviation.

Bacterial growth time	N_0 (number of CFU colonies in a control sample)	N (number of CFU colonies after the treatment)	Log N/N_0
6 h	1.90×10^9	1.02×10^9	-0.27 ± 0.10
11 h	1.66×10^9	8.15×10^8	-0.31 ± 0.03
17 h	1.67×10^9	1.01×10^9	-0.22 ± 0.04

4. Conclusions

Extraction of proteins by means of electroporation 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. In order to optimize the protocol, however several parameters need to be considered. In a previous study we showed that efficiency of extraction of proteins strongly depends on electric pulse parameters [21]. In this study we incubated cells at different temperature before and after electroporation and used cells in different bacterial growth phase in order to determine their effect on the yield of extracted proteins. Our main conclusion is that if we increase the incubation temperature after electroporation, lower protein leakage from bacterial cells occurs. Therefore lower post treatment incubation temperatures are preferred for better protein yield. Our study also showed that bacterial growth time has no effect on protein extraction at low incubation temperatures which yielded highest protein extraction in first part of the study. Since our findings on this matter are not directly comparable to other studies and appear even contradictory, generalization at this stage is not possible and further experiments are needed.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bioelechem.2016.08.002.

Acknowledgments

This research was supported under various grants by the Slovenian Research Agency (P2-0249, J7-6783) and was conducted within the scope of the EBAM European Associated Laboratory (LEA). This research was possible as a result of networking efforts within COST Action TD1104 (www.electroporation.net) and short-term scientific missions (COST-STSM-ECOST-STSM-TD1104-290314-041252) to S.H.M. The authors declare that there are no conflicts of interest. Experimental work was performed in the infrastructure center Cellular Electrical Engineering IP-0510 at University of Ljubljana.

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