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Short communication

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

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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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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.