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Evaluation of a new method for genetic modification of microorganisms

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Avaliação de um novo método de modificação genética de microrganismos

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Avaliação de um novo método de modificação genética de microrganismos

dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica do Dr. Jorge Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro e do Prof. António Carlos Matias Correia, Professor catedrático do Departamento de Biologia da Universidade de Aveiro

to my mum, dad and sister – for the times I was not beside

o júri

presidente

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palavras-chave

bacteria, *Escherichia coli*, plasmideo, pUC19, modificação genetica, transformação, alta pressão hidrostatica

resumo

Actualmente, a tecnologia de DNA recombinante está presente de modo substancial nas nossas vidas, levando as suas possíveis aplicações simultaneamente a esperança e o medo. A transformação genética, mesmo que realizável com várias técnicas, enfrenta ainda eficiências insatisfatórias em muitos casos e impossibilidade noutros. Neste trabalho, foi realizada uma tentativa para estudar o potencial de um novo método de transformação genética, concretamente de introdução do material genético na célula a transformar.

A ideia para este trabalho foi originada da área de conservação dos alimentos, onde a alta pressão hidrostática é utilizada para inactivar microrganismos, principalmente pelos seus efeitos destrutivos sobre as membranas, por exemplo produzindo poros. Deste modo, é razoável supor que nas células stressadas sub-letalmente se criem poros permitindo a introdução de DNA. Assim, para que esta metodologia funcione, um compromisso entre stress suficiente e a manutenção da viabilidade das células deve ser utilizado.

Neste trabalho, a avaliação efectuou-se com o hospedeiro bacteriano - *Escherichia coli* TOP10 e com um pequeno plasmídeo circular – pUC19, que confere uma resistência à ampicilina como marcador. A electroporação, usada como técnica da referência, resultou em taxas de eficiência de $(9{,}47\pm2{,}00)\times10^7$ e $(6{,}30\pm0{,}83)\times10^7$ transformantes/µg de DNA quando foi aplicada concentração de plasmídeo 0,01 µg/mL e de $(1{,}18\pm0{,}37)\times10^7$ e $(3{,}44\pm0{,}56)\times10^7$ transformantes/µg com 100 µg/mL de plasmídeo.

Relativamente à pressão, os tratamentos situaram-se entre 50–400 MPa e o tempo variou dos 10 segundos até aos 5 minutos. Além disso, as pressurizações incluíram um estudo de dois valores da taxa de compressão: 5 e 10 MPa/seg e dois tipos de ciclos: singulares e triplos.

Os tratamentos sob 50 e 100 MPa durante 2,5 e 5 min, assim como as pressurizações sob 200, 300 e 400 MPa durante 1 e 5 min, causaram uma redução da viabilidade ≥99,99% da população bacteriana inicial. Portanto, os valores dos potenciais factores de stress foram limitados a 50–200 MPa, até 1 minuto com uma taxa da compressão 5 MPa/seg, com a redução da viabilidade de aproximadamente 90%. Os tratamentos mais longos realizaram-se com os ciclos múltiplos resultando num número de sobreviventes aproximadamente 3% mais alto, para células pressurizadas sem o plasmídeo. Concluiu-se que *E. coli* pode precisar de algum tempo para recuperar, pelo que é preferível adicionar o meio enriquecido não imediatamente depois uma pressurização, embora as variações não sejam muito significativas. Na maioria dos casos, a adição de plasmídeo reduziu o número dos sobreviventes detectados.

Algumas das experiências resultaram num inesperado mas evidente aumento do número das células, até valores 7,5 vezes mais altos para o tratamento sob 100 MPa durante 30 seg com uma taxa da compressão normal e com adição de pUC19. Estas observações podem dever-se à possibilidade da pressão poder causar desagregação de células ou a indução de germinação.

Com os resultados obtidos não se observou transformação genética com sucesso. Como potencialmente existem vários parâmetros da metodologia (p. ex. vários conjuntos hospedeiro - plasmídeo, indução das competências celulares, as formas e tamanhos de DNA), uma nova futura avaliação da alta pressão para modificação genética deve ser tentada.

keywords

bacteria, *Escherichia coli*, plasmid, pUC19, genetic modification, transformation, high hydrostatic pressure

abstract

Nowadays recombinant DNA technology is broadly present in our lives, bringing both hopes and fears. Transformation, even if achievable by several techniques, still faces with unsatisfying efficiencies and lack of universality. In this work, an attempt to evaluate the potential of a new transformation methodology was made.

The idea for this work came from food preservation where high hydrostatic pressure is utilized to inactivate microbial flora, mainly by destructive effects on membranes, as for instance cavities. This way, it is rational to think that sub-lethally affected cells would create pores allowing the uptake of DNA. Thus, in order to compel the method to work, a compromise between enough stress and maintenance of some cells still viable and capable to recover must be looked for.

In this work, the assessment was performed on bacterial host - *Escherichia coli* TOP10 and small circular plasmid – pUC19 that provides resistance to ampicillin as a selection marker. Electroporation, the reference technique, resulted in transformation efficiency rates of $(9{,}47 \pm 2{,}00) \times 10^7$ and $(6{,}30 \pm 0{,}83) \times 10^7$ transformants/µg of DNA when 0,01 µg/mL plasmid was applied and of $(1{,}18 \pm 0{,}37) \times 10^7$ and $(3{,}44 \pm 0{,}56) \times 10^7$ transformants/µg of DNA with 100 µg/mL plasmid (double values are derived from assays carried at distinct days).

Concerning pressure, treatments were limited to 50-400 MPa and the time ranged from 10 seconds to 5 minutes. Pressurizations included also examination of two values of compression rate: 5 and 10 MPa/sec and two cycle variants: single and triple.

Treatments under 50 and 100 MPa during 2,5 and 5 min, as well as pressurization under 200, 300 and 400 MPa during 1 and 5 min, caused reduction of viability ≥99,99% of initial bacterial population. As a result, values of the potential stress factors were narrowed to 50–200 MPa lasting up to 1 minute at 5 MPa/sec compression rate, with the reduction of viability approximately 90%. Longer treatments were performed with multiple-cycles resulting in number of survivors of approximately 3% higher values (for cells pressurized without plasmid). It was concluded that *E. coli* may require some time to recover and so rich nutritionally broth should not be added immediately after pressurization, although fluctuations were not of great significance. In majority of the cases, addition of plasmid reduced the number of detected survivors but the variations were almost unnoticeable being in the range of few percent.

Some of the experiments resulted in surprising but evident increase of cell number, reaching even up to 7,5-fold increase for the treatment under 100 MPa during 30 sec at normal compression rate with addition of pUC19. Such observations were supposed to origin from disaggregating or germination-inducing activity of pressure.

With the results obtained, no successful transformation was observed. Since there exist several potential improvements of methodology (i.e. various host-plasmid sets, induction of cell competences, forms and sizes of DNA) future evaluation could hopefully bring positive results.

Index of content

List of figures IV

List of graphs VI

List of tables VII

Abbreviations / Nomenclature VIII

1. Introduction 1

1.1. Genetic Engineering 1

1.1.1. Why genetically modify microorganisms? 1

1.1.2. How to genetically modify the microorganisms? 2

1.1.3. Transformation - which methodology is the best? 3

1.1.4. The host - why *Escherichia coli* TOP10? 6

1.1.5. The vector - why pUC19? 8

1.2. Food Preservation 11

1.2.1. What is `food preservation`? 11

1.2.2. What are the techniques for preserving foods? 12

1.2.3. What is the role of high hydrostatic pressure in food preservation? 13

1.3. High pressure and microorganisms 15

1.3.1. What are the governing thermodynamic laws? 15

1.3.2. What is the impact of HHP on cell envelopes? 16

1.3.3. How does HHP affect proteins? 18

1.3.4. How does HHP influence enzymatic activity? 21

1.3.5. What is the effect of HHP on ribosomes? 22

1.3.6. How does the HHP alter the nucleic acids? 22

2. Materials and Methods 24

2.1. Microorganisms 24

2.1.1. Electrocompetent cells 24

2.1.2. Non-electrocompetent cells 25
2.2. Plasmid 25
2.2.1. Commercial pUC19.....25
2.2.2. Extracted pUC19.....25
2.2.3. Quantification of extracted plasmid 25
2.3. Genetic transformation 26
2.3.1. Electroporation.....26
2.3.2. Innovative methodology 27
2.4. Methods of quantification.....27
2.4.1. Quantification of microorganisms.....27
2.4.2. Quantification of transformants 28
2.4.3. Efficiency of transformation 28
2.5. Discrimination between transformants and contaminants.....29
2.5.1. Expression of β-galactosidase.....29
2.5.2. Microscopic observation.....29
2.5.3. Plasmid extraction 29
2.5.4. Gram staining.....29
2.6. Culture media and asseptic methods 30
2.6.1. LB and LA medium.....30
2.6.2. SOC broth 30
2.6.3. The asepsis.....30
3. Results and Discussion 32
3.1. Transformation with conventional methodology.....32
3.2. Transformation with an innovative methodology.....32
3.2.1. Optimization of treatment time 32
3.2.2. Optimization of pressure value 33

3.2.3. Optimization of inactivation level.....35
3.2.4. Evaluation of increased concentration of plasmid 41
3.2.5. Impact of single- and multiple-cycles 46
4. Conclusions and Proposals for future 54
Bibliography.....56
Appendices.....59

List of figures

Figure 1 Genetic map of the pUC19 plasmid, adapted from New England Biolabs [30].....10

Figure 2 Schema showing mechanism of membrane permeabilization during high pressure treatment, adapted from Gänzle, 2001 [51].....17

Figure 3 Impact of pressure on polypeptides, adapted from Aertsen, 2009 [2].....20

Figure 4 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Blank i.e. not pressurized sample of *E. coli* and pUC19 (0,1 MPa, ∞); Volume plated: 80µL.....43

Figure 5 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 50±5 MPa, 30 sec.; Volume plated: 40 µL (left plate) and 80 µL (right plate).....43

Figure 6 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 100±5 MPa, 30 sec.; Volume plated: 40 µL (left plate) and 80 µL (right plate).....44

Figure 7 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 200±5 MPa, 10 sec.; Volume plated: 40 µL (left plate) and 80 µL (right plate).....44

Figure 8 Results of blue/white screening of 20 morphologically diverse colonies. Petri dish: LA, ampicillin, X-Gal and IPTG; Incubation: 24 hours, 37ºC. 46

Figure 9 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Blank i.e. not pressurized *E. coli* and pUC19 sample transferred to 400 µL Eppendorf tube (0,1 MPa, ∞); Volume plated: 40 (left plate) and 80 µL (right plate). 48

Figure 10 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 100±5 MPa, 60 sec., 1 cycle; Volume plated: 40 µL (left plate) and 80 µL (right plate).....49

Figure 11 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 100±5 MPa, 60 sec., 3 cycles; Volume plated: 40 µL (left plate) and 80 µL (right plate).....49

Figure 12 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 100±5 MPa, 150 sec., 1 cycle; Volume plated: 40 µL (left plate) and 80 µL (right plate).....50

Figure 13 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 100±5 MPa, 150 sec., 3 cycles; Volume plated: 40 µL (left plate) and 80 µL (right plate).....50

Figure 14 Non-selective Petri dish (LA) after 48 hours of incubation at 37ºC; Samples of distilled water subjected to standard experimental procedure for blanks – not pressurized; Volume plated: 100 µL.....52

List of graphs

Graph 1 Results of cell viability after treatment depending on stress factor (Pressure*Time). 33

Graph 2 Decimal reduction of cells caused by treatment depending on stress factor (Pressure*Time). 34

Graph 3 Results of cell viability after treatment depending on stress factor (Pressure*Time*Compression rate). 35

Graph 4 Results of cell viability depending on treatment time. Data for normal compression rate. 36

Graph 5 Comparison of influence of compression rate on cell viability after treatment depending on pressure. Data for treatments at 50, 100 and 200 MPa during respectively 30, 30 and 10 seconds. 37

Graph 6 Results of cell viability after treatment depending on pressure. Data for pressurizations at normal compression rate (approximately 5 MPa/sec.) during 30 seconds. 38

Graph 7 Results of cell viability after treatment depending on stress factor (Pressure*Time*Compression rate). Data for samples with SOC supplemented after delay (app. 10 minutes). 39

Graph 8 Results of cell viability after treatment depending on stress factor (Pressure*Time*Compression rate). Data for samples with SOC supplemented immediately. 39

Graph 9 Inactivation of treated cells during the 0- and 2-day storage at $4^{o}C$ depending on applied stress factor (Pressure*Time*Compression rate*Days of storage at $4^{o}C$). 40

Graph 10 Results of cell viability after treatment depending on stress factor (Pressure*Time*Plasmid). 41

Graph 11 Decimal reduction of cells caused by treatment depending on stress factor (Pressure*Time*Plasmid). 42

Graph 12 Results of cell viability after the treatment depending on stress factor (Pressure*Time*Nº of cycles*Plasmid). 47

List of tables

Table 1 Available technologies of transformation and their evaluation [9, 11-18] ...4

Table 2 Positive and negative aspects of food processing [35, 36].....12

Table 3 Impact of high pressure on chemical bonds due to the volume change, adapted from Rivalain, 2010 [49].....16

Table 4 The content of a rich SOC broth.....30

Table 5 Results of transformation efficiency for electroporation of electrocompetent *E. coli* TOP10 with average values and standard deviation (STDV).....59

Table 6 Results obtained after treatment of non-electrocompetent *E.coli* TOP10 with stress factor (Pressure*Time): cell counts, viability and decimal reduction. 59

Table 7 Results obtained after treatment of non-electrocompetent *E.coli* TOP10 with stress factor (Pressure*Time*Compression rate): average values of cell counts and viability and their standard deviations (STDV).....59

Table 8 Results obtained after treatment of non-electrocompetent E.coli TOP10 with stress factor (Pressure*Time*Plasmid): average values of cell counts, viability and decimal reduction with standard deviation (STDV). 59

Table 9 Results obtained after treatment of non-electrocompetent *E.coli* TOP10 with stress factor (Pressure*Time*Nº of cycles*Plasmid): average values of cell counts, viability and decimal reduction with stndard deviation (STDV). 59

Abbreviations / Nomenclature

Abbreviation	Designation	Units
A	Absorbance	-
Amp	Ampicillin	-
EDTA	Ethylenediaminetetraacetic acid	-
E_t	Transformation Efficiency	Transformants/µg of DNA
FCR	Fast Compression Rate	-
GEM	Genetically Engineered Microorganisms	-
GMO	Genetically Modified Organisms	-
HCl	Hydrogen Chloride	-
HHP	High Hydrostatic Pressure	Pa
IPTG	Isopropyl-β-D-thiogalactopyranoside	-
LA	Luria Agar media	-
LB	Lysogeny Broth	-
NaCl	Sodium Chloride	-
NCR	Normal Compression Rate	-
OD	Optical Density	-
PHA	Polyhydroxyalkanoates	-
SOC	Super Optimal Broth	-
TE	Tris-EDTA	-
X-gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside	-

1. Introduction

1.1. Genetic Engineering

1.1.1. Why genetically modify microorganisms?

Microorganisms are broadly present in human lives, bringing both benefits and harm. Thus, possible improvements and innovations that could increase or induce their utility are uncountable. Genetic engineering delivers an efficient source of solutions. Modifications of DNA may enrich organisms in various features, for example by elimination or addition of specific genes, enhancement of gene expression, or redesign of some sequences. [1, 2]

Two of the most important aspects of human live: health and aliment, profit nowadays from genetically modified microorganisms (GMOs or GEMs). They provide production of pharmaceutics (e.g. insulin, vaccine against hepatitis B) achieving competitively high yields of processes. Furthermore, since traditional manufacture of medicines faces safety hazards, risk-free (non-pathogenic) application of GEMs serves as valuable alternative. Still, success of the treatment depends on fast and accurate disease recognition. Some of diagnostic immunologic kits (for AIDS or Alzheimer´s disease) utilize products expressed by genetically engineered microorganisms. [1, 3]

GEMs are present in food industry supplying companies in various compounds and additives that enrich composition and develop novel attributes of products. Thus, they provide tools permitting manipulation of food flavor, aroma, tint and structure. More, they contribute to more clean and efficient manufacturing procedures. Traditional processes are replaced with more effective solutions and beneficial from an economic, energetic and environmental point of view. Mentioned advantages are valid also for other industrial applications of genetically modified microorganisms like textile or paper production. [1, 4]

In the animal farming, supplements like growth hormones derived from recombinant microorganisms are utilized in feeding. In agriculture influence of pathogenic factors and harmful action of insects in plant breeding may be limited by GEMs. Simultaneously substitution of chemicals by naturally delivered compounds eliminates risk of toxicity for consuming organisms. [1, 3]

As rather novel and emerging, environmentally-friendly solutions provided by genetically engineered strains deserve special attention. Those microorganisms are capable to perform bioremediation. Thus, their activity can significantly contribute to reduction of pollutant content in environment. Another useful and valuable GEMs attribute is production of polyhydroxyalkanoates. Naturally created PHA polymer is biodegradable and may successfully substitute traditional plastics. [1, 5]

1.1.2. How to genetically modify the microorganisms?

Applications described in previous paragraph are achievable owe to the recombinant DNA technology. It would be impossible to realize without essential tools - enzymes. Among numerous others, restriction endonucleases and ligases are of special importance. All techniques of genetic modification follow the same general scheme briefly described below. [6, 7]

The targets of gene cloning refer always to some particular part of genetic information. Hence, primarily gene or DNA fragments of interest must be generated. This step includes DNA extraction from the donor organism and isolation of specific sequence. The latter objective is completed by irreplaceable activity of restriction endonucleases. Those are highly specific enzymes that hydrolyze the deoxyribonucleic chain. They recognize restriction sites usually consisting of 4-6 base pairs in the surrounding of the fragment of interest. Next, they cut the chain creating blunt or sticky ends with terminal 5’-phosphates. Cohesive tails are preferred because they facilitate subsequent ligation with vector. [6, 7]

Second step encompasses insertion of isolated DNA fragment to the vector or carrier molecule that allows the efficient amplification and expression of the sequence in a host cells. That is done by enzymes able to perform processes that reverse the result of digestion with restriction endonucleases. The vector should be formerly prepared and cut with restrictase in order to form ends complementary to those found in insert. Tails of both: isolated sequence of interest and vector, are hybridized and covalently joined by DNA ligase. The enzyme creates phosphodiester ligations between 5’-phosphorylated and 3’-hydroxylated terminals. [8]

Finally, modified molecule is introduced into the host by a process called transformation. Unfortunately, it still remains an unfulfilled wish to transform all cells

present in the suspension. When the process is performed applying ligation mixture, three types of cells are obtained: without the carrier molecule, with non-recombined vector and with recombined vector. The last-mentioned, as successfully modified are named ‘transformants’ and they need to be verified in a subsequent step. [6]

The presence of introduced vectors is determined through selection. Carrier molecules are typically provided with a selectable marker that permits easy detection of transformed cells. The gene usually encodes resistance for substances normally toxic for the hosts or enriches them in the ability to grow in specific incubation conditions. Thus, cells after transformation are plated on medium containing the composite for which they gained resistance incorporating vector. In the effect, growth of non-transformants that are sensitive for the factor is inhibited. [6, 8]

Still, recognition of cells carrying vector with and without insert of interest is required. There are numerous available screening methods based on analysis of functionality or sequence of DNA fragment, for example plates with chromogenic substrates or PCR. Screening is not necessary in the case when vector solution utilized in transformation step contains only recombined molecules. [6, 8]

1.1.3. Transformation - which methodology is the best?

The concept of “transformation” implies introduction of “naked” DNA into a cell. It happens naturally or with human help. In the first case, plasmid or any DNA fragment liberated by dead cells can be incorporated by bacteria. Those microorganisms are defined as “naturally competent”. The man takes advantage of transformation and applies it in the recombinant DNA technologies holding in the result wild range of techniques. [7, 9, 10]

Table 1 [9, 11-18] provides graphical overview on available technologies, their principles of action, typically achieved efficiency rates and general evaluation.

Table 1 Available technologies of transformation and their evaluation [9, 11-18]

The efficiency of transformation may be improved if pre-prepared host cultures are utilized in the process. There are various techniques increasing fragility of the cells and in the meantime - their membranes. In majority they include additives such as glycine, antibiotics or enzymes. Since those extra-composites are usually toxic they must be applied with special attention. However, introduction of another step to procedure increases value of time- and work-consumption. [9, 13]

The process of transformation includes three phases:

- Membrane permeabilization - cells are made competent through weakening the external envelopes.
- Stress - cells are subjected to the factor introducing DNA inside the host.
- Recovery - temporary damages are repaired and cells regain growth abilities. [9]

The most serious challenge to overcome is still considered induction of outer membrane semi-permeability. Cell envelopes may not allow transport of macromolecules carrying a large electrical charge (like DNA). In general, all transformation methods need to be optimized by finding and reaching the equilibrium between membrane destruction and cell viability. [9, 19]

1.1.4. The host - why *Escherichia coli* TOP10?

Selection of the host for particular genetic engineering experiment must be adjusted to the final purpose of practice. Nevertheless, there may be specified some general attributes that should characterize ideal host organism. It needs to provide the operator with easy manipulation and propagation procedures. At the same time, the host species should be diverse enough to contain strains with different properties and capabilities. It is also desirable to be efficient in work with various DNA carriers. [6]

The most successfully and extensively applied host organism is *Escherichia coli*. It represents all characteristics listed above and what is more, has other advantageous features. In majority, this bacteria results in highly efficient practices of recombinant DNA technology. As an object of numerous investigations it became well understood and described living organism. Projects of genetic maps brought knowledge of entire chromosome sequence. Finally applications of *E. coli* are cost effective permitting taking advantage of still economic scaled-up productions. [20]

Escherichia coli are gram-negative bacteria naturally present in the intestinal tract providing warm-blooded animals with vitamins and preventing growth of pathogens. Therefore, most of the species does not cause diseases. It is able to grow in both - aerobic and non-aerobic environments. As a prokaryotic organism it has a haploid circular chromosome organized in nucleoid structure. Although highly conservative, the genome of *E. coli* varies in size among species, containing on average 4-5x10^6 base pairs. Importantly, gene expression proceeds rather quickly and without post-transcriptional processing what could be troublesome in some cases.

The TOP10 Electrocompetent *E. coli* cells were purchased from Invitrogen. [21] According to the provided by the company manual, cells - as competent - are fragile for the temperature and mechanical factors. The genotype of the strain, using proper abbreviations, is described as follow: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ-. [22] It is said to be close to the genome of DH10B *E. coli* strain. TOP10 cells are characterized by some special attributes improving performance of cloning. In particular it is achieved by construction of a genome that contains mutations and deletions reducing natural processes of DNA modification in bacteria, for example disabling systems of repair. [23]

In accordance with description, the TOP10 strain is unable to conjugate with other individuals since it lacks the conjugative F plasmid. Cells do not carry any additional sequence of the phage λ. Mutation *mcr*A contributes to its competence since it eliminates breakage of methylated deoxyribonucleic acid normally identified as a heterologous. The microorganisms are deprived of restriction system (*mrr-hsd*RMS-*mcr*BC) recognizing and digesting exogenous DNA. Thus, this deletion is important to avoid that the *E. coli* host eliminate inserts from other organisms. [22]

The genome of recombinant. *E. coli* permits the screening of successfully transformed bacteria through the α-complementation (guaranteed by φ80*lac*ZΔM15). [24] They carry also mutation in gen RecA avoiding recombination between insert and homologous sequences present in the genome. [25] The TOP10 strain is characterized with increased stability of the DNA because mutation *end*A1 blocks expression of one of endonucleases. [24] The mutation in nupG gene results in disturbed nucleoside transport what could hypothetically contribute to the highly efficient performance of transformation. However the opinions are still contradictory and there are lacks of clear proofs. [22, 26]

In *E. coli* TOP10 part of the lac operon was deleted, thus cannot metabolize lactose. On the other hand, this loss gives opportunity to utilize convenient chromogenic screening methodology when combined with vectors that carry lac gene - details provided later. [23] Mutations introduced in *ara*D, *gal*U and *gal*K genes inactivate enzymes breaking arabinose and galactose molecules preventing their assimilation by bacteria. [22, 27] Furthermore, to grow the strain needs externally delivered leucine being unable to synthesize it. [28] The genome´s characteristics also determine that this strain is stably resistant to streptomycin. [22, 24]

1.1.5. The vector - why pUC19?

The choice of a suitable carrier molecule or vector in the transformation procedure contributes to final success as well. Since large vectors have problems in stability during replication, the ideal vector is minimized in size, thus facilitating its isolation. Obligatory, it must contain an origin of replication (ori site) that guarantees independent copying and stability in the developing population. To help a DNA insertion, vector must carry adequate restriction site. Sequences recognized by restriction endonucleases should be single along the entire carrier molecule. It is convenient if part of a vector encodes selectable marker. This gene usually brings resistance for substances normally toxic for the hosts or enriches them in the ability to grow in specific incubation conditions. [6]

First cloning experiments were performed with bacterial plasmids of *E. coli* as vectors. Those naturally occurring molecules of DNA encompass several advantages that decided about their success. Plasmids are small, extrachromosomal, usually circular DNA with self-replicating capability. They replicate faster than the chromosome and because of that they may exist in several copies in the cell, reaching sometimes even up to a few hundred copies. Longer plasmids tend to replicate slower. As potentially ideal vectors, they have also restriction sites. Moreover, plasmids carry several genes and one of them may encode the resistance for bactericidal substances, becoming simultaneously selective genes. One of the most common resistance genes encodes a β-lactamase, an enzyme that hydrolyses antibiotics from penicillin group (e.g. ampicillin). [10]

To improve application of plasmids in genetic engineering, they have been reconstructed and enriched in valuable attributes. As a result, a set of commercial vectors originating from bacteria was established including among many others, family of pUC.

Those vectors are characterized by higher flexibility, comparing to natural plasmids because they gained an artificial sequence containing multiple cloning sites, called polylinker. This region holds several unique restriction sites allowing integration of inserts generated with different type of restriction enzymes. As another attribute, pUC family involves just part of β-galactosidase gen - fragment responsible for expression of α-peptide. [29]

The sequence coding α-peptide was manufactured in order to contain the polylinker "in frame" what means that the gene is read and can be translated into a functional protein. That is why *E. coli* containing vector is able to hydrolyze X-gal giving rise to blue colonies on medium enriched with the chromogenic substrate. When to the polylinker is ligated insert, the coding sequence is disrupted. Thus, the gene is not able to originate the functional protein and since bacteria cannot metabolize the X-gal growing colonies remain white. [29]

Figure 1 illustrates the genetic map of a constructed plasmid pUC19. [30] It consists of 2,686 base pairs, thus being profitably small. It belongs to the family of high-copy number vector and its particularly designed structure permits the control by temperature of the amount of amplicons present in the cells. Among numerous restriction sites, carries several exclusive and those are typed in bold. Plasmid may be selected due to its resistance to ampicillin. Vector construction allows also screening by α-complementation as described before.

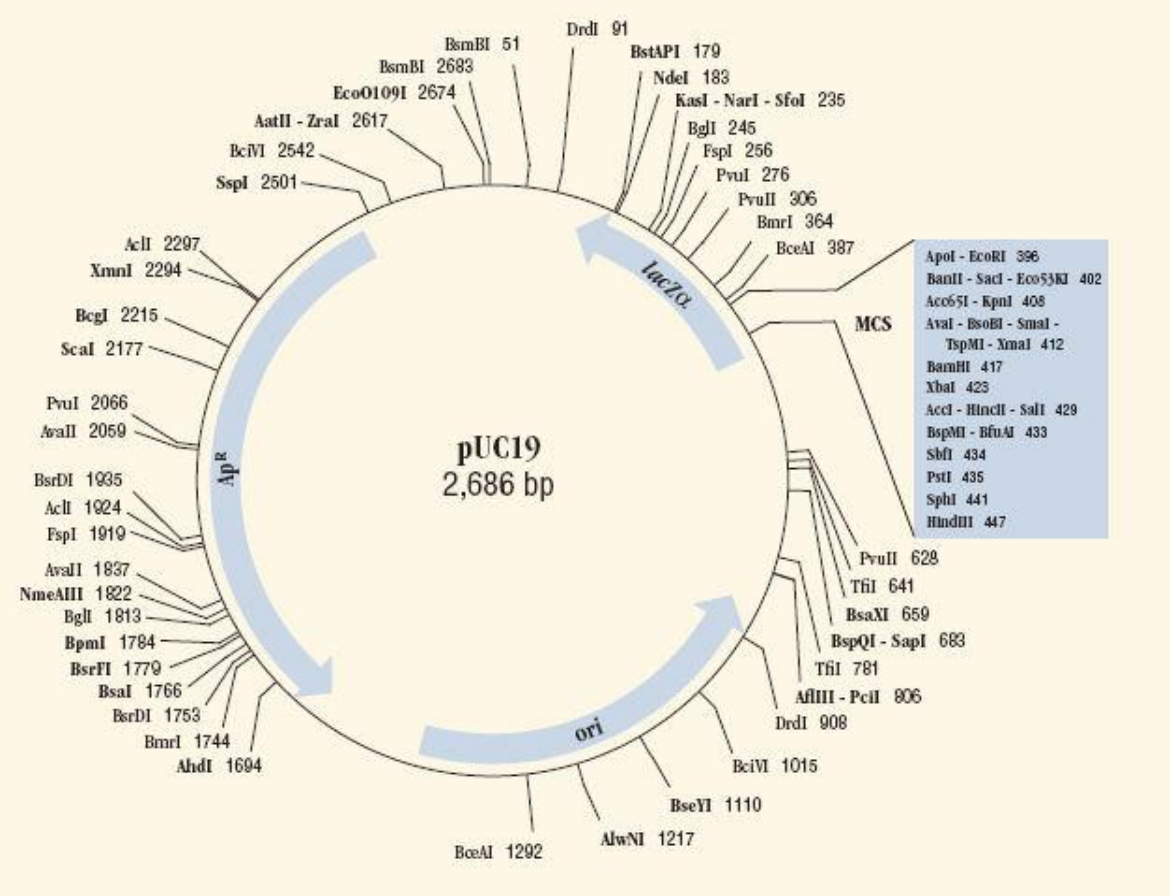


Figure 1 Genetic map of the pUC19 plasmid, adapted from New England Biolabs [30]

1.2. Food Preservation

1.2.1. What is `food preservation`?

The term "food preservation" means all operations that target keeping natural or required characteristics of alimentary products (raw, processed and formulated) for a long time. In other words, it includes possible ways of prolonging shelf life of foods by preventing them from spoiling what significantly reduces general quality.[31]

Conditions during entire processing can influence and induce various mechanisms reducing the nutritional value of foods or even turning them poisonous when absorbed. In this sense "shelf life" is understood as a period of time when product can be transported and stored without loss of desired quality. It lasts till food is no longer recommended to be consumed. [31]

There are many recognized factors contributing to food degradation, for example temperature, light or presence of water. To systematize deterioration agents, they may be classified with regard to their mechanism of actions as:

- microbiological - in addition to pathogenic activity, microbes are able to spoil food, change the content due to their metabolism, production of off-odors, off-flavors etc.;
- enzymatic - reactions causing brown color, bitter or fishy flavors etc.;
- chemical - interactions between composites and with environmental compounds (e.g. oxidations, lipolysis, discoloration);
- physical - for example phase alterations during thawing and refreezing (ice-cream gains sandy structure);
- mechanical - injuries, caused usually by mishandling, interrupt first protective barrier (e.g. skin or shell) and break natural "security systems". In the effect, products are facilely targeted by other factors. [31, 32]

Of those listed above, inactivation of microorganisms seems to be a primary target in food preservation since products released to the market, due to the law, must be stable and safe for consumers. [33]

The following table 2 shows main advantages and disadvantages of food processing. Nowadays, in order to fulfill consumers' demands, the effort is put on

optimization of applied methods to raise their beneficial effects and minimize the invasiveness.[34]

Table 2 Positive and negative aspects of food processing [35, 36]

Advantages	Disadvantages
• Elimination of microorganisms (health and quality) • Destruction of naturally occurring anti-nutritional compounds (e.g. toxins and enzymes) • Reduction of unwanted residues acquired from environment (e.g. pesticides) [37] • Prolongation of shelf-life • Nourishing aspect (facilitated digestion and delivery of nutrients) • Quality enrichment (taste, flavor, texture) • Compositional improvement (presence of probiotics, antioxidants etc.) • Economy of time, work and money • All-year availability	• Undesired waste of components • Development of neutral or harmful compounds (e.g. trans fatty acids, glucoinolates) • Quality reduction

1.2.2. What are the techniques for preserving foods?

The history of food processing is said to set up approximately 700 000 years ago with early (not completely aware) thermal techniques. Since then, people invented and developed more complex and sophisticated methods, becoming also more aware of their purpose. Special advances came after the Second World War when processing of food was scaled-up and reached industrial level. [35]

In effect, gradual progression drove the way to today available techniques. Frequent experiments and studies resulted in extensive knowledge and data that allow to control those processes more accurately, determining precisely final content and quality of a product. [35, 36]

According to the review by Floros [36] among numerous typical techniques we can find: mechanical operations, heating (including for example pasteurization or blanching), refrigeration and freezing, dehydration, acidification, fermentation, reduction of water activity, smoking, irradiation, extrusion, modified/controlled atmosphere, additives and packaging. However, increasing demands of customers for further improvements and novel promising processes appeared in the doorstep. As a principle they shorten the time and/or

lower the temperature of treatment. They include microwave and ohmic heating, high pressure and pulsed-electric fields. Those solutions still require more extend assessment to be approved by legislation and successfully introduced in the industry, with the exception of high pressure.[36]

Another opportunity was brought by the idea of hurdle technology. It takes simultaneously advantage of several available methods resulting in safe and stable processed products but still fresh. In the concept of hurdle technology is creation of such a set of barriers that in the final effect it is impossible for any microorganism to overcome. Since several stress factors are mixed in a single technique, having synergistic effect, they do not need to work at extreme conditions - treatment may be much less intense. All those circumstances cause hurdle technology to be favorably applied in the industry. [35, 38, 39]

1.2.3. What is the role of high hydrostatic pressure in food preservation?

Even though Bert Holmes Hite published the results of his work on preservation of milk by high pressure already in 1899, this method belongs to one of the most recent. Just during last 20 years the idea came out of the laboratory to industry and to markets becoming competitive option for the heat treatment, from economical and technological points of view. [40, 41]

High hydrostatic pressure guarantees microbiological safety of products and accompanying prolongation of their shelf-life. Its antimicrobial action is complex and multi-target. Cell membranes, proteins, enzymatic activity, ribosomes and nucleic acids are affected in the process. Inactivation encompasses vegetative pathogens and also spores. The latter ones are more resistant and because of that in some cases it may be required to combine pressurization with other mild treatment (e.g. heat) to obtain sufficient growth inhibition. Viruses, prions, several enzymes and allergens are possible to be eliminated as well. [35, 42, 43]

What is more, contrary to thermal processes, synthesis of unwanted composites does not occur. The aspect of formed by-products that are harmful for health was for many years serious argument against preservation of food. [35]

Pressure treatment successfully meets requirements of customers since it maintains foods fresh and minimally affected. Furthermore, loss of nutritionally valuable compounds is significantly reduced. Considering pressurization in terms of transferred energy,

it is not able to break bonds in small molecules like vitamins or amino acids. [31, 35, 41, 42]

During pressurization foods do not change the physical structure, maintaining organoleptic character because share forces are not induced. Possibly they gain beneficial properties of texture due to complicated modifications of interactions and compounds. For example, increased softness of fruit tissue and intensified viscosity of milk were observed after pressure treatment. More, foods in great part retain their color and flavor compounds. Advanced pressurizing equipment available on the market allows precise manipulation of treatment conditions, what in turn gives strict control of texture and quality. [35, 41, 42]

Another advantage characterizing this technique of preservation is broad range of products to which it may refer. Current applications include among others: juices, fruit, vegetable or meat. It also provides alternative for products requiring low-temperature processing. [41, 42]

From a practical point of view, pressurization is also a convenient technology because it can be applied to already packaged products. Up to the moment when consumer purchases and opens the object, the entire unit maintains safe quality. Moreover, shape and size of treated objects are restricted only by the capabilities of the equipment that is utilized. [35, 41, 42]

Food preservation by high hydrostatic pressure gains appreciation as a technique safe for environment, free of waste residues production. [42]

Apart from all advantages over thermal processes, still, high hydrostatic pressure faces some drawbacks. One of them is lack of formation of a flavor characteristic in case of heat treatment. Besides, it is a solution requiring high initial capital, what is problematic for most of the companies. Also the packaging material is a challenge - it must possess specific features like compressibility, resistance or stability of composition. [35, 41, 42, 44]

1.3. High pressure and microorganisms

1.3.1. What are the governing thermodynamic laws?

The impact of high pressure on molecules and their reactions may be generally described with the Le Châtelier´s principle. In accordance with it:

If a system at equilibrium is disturbed, a reaction will occur which will reduce (but not eliminate) that disturbance and create a new, shifted, equilibrium. [45]

Considering pressure, a reaction will be accelerated if the volume of the system after reaction (products + solvent) will be lower than before the reaction (reactants + solvent). Contrary, when the volume of system increases along the process, reaction will be decelerated. [2, 46]

However, the Le Chatelier´s principle as the majority of recognized thermodynamic laws relates to systems in equilibrium and there exists several evidences stating nonequilibrium character of biological systems. In effect, responses of living cells to environmental factors are more complex and more drastic than *in vitro* models. [2, 47]

Working in adiabatic conditions, it is important to have on mind temperature changes during compression and decompression. Increase of pressure causes heating effect in treated systems and diminution – inversely - cooling. Analyses show that temperature can raise 2 - 3ºC every 100 MPa in aqueous environment, what is significant. Moreover, it depends also on detailed features of treatment applied - rate of pressure change and specific nature of the sample. [2, 48]

High pressure treatment should be also identified with regard to energy conveyed to the system. Narrowing pressure variation to 0,1 - 1500 MPa, the range of transmitted energy is few kJ/mol. It is far too less to alter stronger molecular interactions like covalent bonds. [2]

Table 3 presents chemical interactions, volume changes accompanying their breakage and possible pressurization effect. According to it, hydrophobic, as well as ionic bonds, are the most susceptible ones. [49]

Type of interaction	$\Delta V_{dissociation}$ (ml mol^{-1})	Pressure effect
Covalent	+10	Stabilization
Ionic	−10	Destabilization
Hydrogen	+3 to −1	Stabilization or low destabilization
Hydrophobic	<0 (−10 to −20)	Destabilization

Table 3 Impact of high pressure on chemical bonds due to the volume change, adapted from Rivalain, 2010 [49]

1.3.2. What is the impact of HHP on cell envelopes?

Escherichia coli, like other microbes, are protected from influences of environmental factors by a lipid membrane. Due to amphiphilic nature, phospholipids are able to spontaneously form bilayer structures in the presence of water. That is how majority of bimolecular envelopes is constructed: hydrophobic rests of fatty acids are isolated from aqueous environment by hydrophilic parts of molecules. [49, 50]

Superficial layer of cell membrane contains lypopolisaccharides with several hydrocarbon chains. Those chains may hold saturated or unsaturated bonds. The latter form a bend structure and so "twisted" fatty acids occupy more extended space than straight ones. As a result, membranes with the high percentage of saturated components are stiffer. Piezophilic microbes are characterized by more flexible envelopes that can resist natural pressure stress. [49, 51, 52]

It is proved that under pressure stress liquidous character of biological membranes decreases. Acyl groups gain more straight structure followed by noticeable alterations as sideway thickening and intensification of density. Additionally, phase change takes place and membranes switch their liquid-crystalline nature to gel. [49, 50, 53]

Membranes are not only mechanical barriers isolating cells from environment. They take part in numerous vital processes, like production of energy, osmosis, reaction and communication with external environment. Thus, any disturbance of membrane can easily affect those actions, possibly inhibiting or activating them (only in mild stress). For example, osmotic gradient can be interrupted since bacteria may not be able to control differences in concentrations of solutions inside and outside the cells. Activity of several living systems that are integrated with membranes, like some enzymes or transporting

proteins, may be reduced or totally eliminated when the molecules are detached. The level and manner of porins expression is also altered. [50, 51]

High pressure brings temporary sensitivity to antibiotics and other antimicrobials. Resistance is usually regained when stress factor is eliminated. Process is still unclear and because of that requires further experiments. The possible explanation could be found in the mechanism of inactivation. Negatively charged groups (phosphate, carboxyl) of polysaccharides are able to fix lethal compounds preventing them from action. Permeabilization of membrane causes microorganisms incapable to bind them on surface and resistance is lost. [51, 54]

On the other hand, in the review by Masschalck [55] contrary hypothesis of `pressure promoted uptake` is suggested. Likely, lipopolysaccharides in high pressure-rearranged membrane bind positively charged composites - for example nisin. Tied molecule is transported through outer bilayer and delivered inside the cell. [55]

The hypothetical process of membrane permeabilization by high pressure may be visualized as on figure 2. [51]. According to the authors, pressurization causes phase transition of the phospholipids and ions deprivation. In effect, reversibly permeable cell envelopes are observed. Since this state lasts just for seconds, temporary alterations in structure depend only on value of applied pressure. [51]

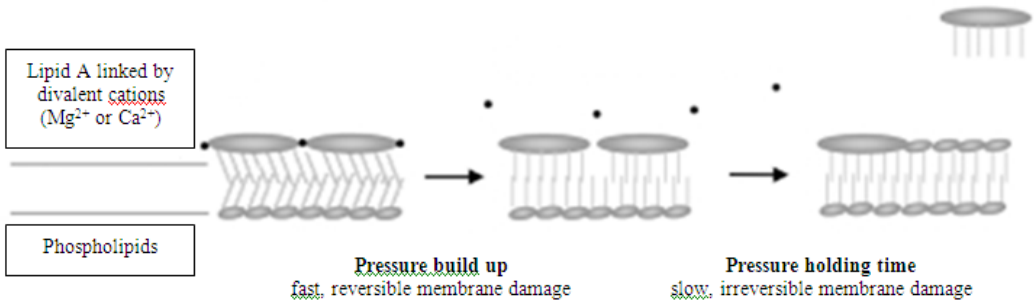


Figure 2 Schema showing mechanism of membrane permeabilization during high pressure treatment, adapted from Gänzle, 2001 [51]

Maintaining the pressure stress for longer time, membranes follow more severe modifications that cannot be regenerated. Molecules of lipid A are successively detached during the treatment and so state of irreversible changes depends not only on value but also on time of pressurization. [51]

Pilar Mañas [56] observed visible changes in exponential-phase cells treated with pressure. They noticed:

- vesicles which are likely formed by lipids released from external membrane,
- areas of thickening placed in cytoplasm, probably created from material of disorganized cell membrane,
- invaginations-like structures toward the inertial part of cell.

It is suggested that vesicles may be produced as a result of different capabilities of outer membrane and internal water to compress.[56]

In pressure treatment, firstly deprivation of fluids from sacs may be observed along the compression. Secondly, while decompression, as a consequence of membrane abundance and decreased filling, shape of outer layer alternates and buds are formed. Regard to this theory, similar modifications of envelopes cannot be noted in stationary-phase cells since their membranes posses lower ability to compress. [56]

In some experiments cytoplasmic macromolecules came out from pressure-treated microbes. This fact proves that created membrane interruptions must have been sufficiently spacious to allow leakage of particles like for example RNA or cellular proteins. [43, 56]

However, death of microorganisms in technologies based on pressure is not entirely induced by membrane injury. Certainly the cell envelopes are the first and primary object in pressurization but it is multi-target and complex process of inactivation. Other alterations in living cells must be taken into consideration since they are involved in microbial death. [49, 53, 56]

1.3.3. How does HHP affect proteins?

The impact of high pressure on proteins can be understood with regard to thermodynamical considerations shortly presented in the part 1.3.3. As mentioned before, high pressure transmits quite low energy and because of that can affect only weak chemical bindings. The higher value of stress applied in pressurization, the more severe are alterations observed in molecular structure. [2, 49]

Native molecules of polypeptides display biological activity due to the proper folding. The conformation is maintained only if all stabilizing and destabilizing interactions affecting the molecule are in balance. Set of internal and external interactions

involving polypeptide and solvent particles provides secondary, tertiary and quaternary conformation of protein. [57]

Mechanism of protein denaturation by pressure differs from that caused by temperature or chemicals. Contrary to other factors, pressurization can save conformation of several fragments of polypeptide molecules, for example some β-like structures. Obviously the difference occurs due to the low amount of transferred energy. [49]

Structural changes of proteins may be classified due to the range of pressure applied:

- < 150 MPa - change of quaternary conformation. Since this level of conformation depends mostly on hydrophobic interactions (the most sensitive), already mild treatment may disturb it. On this stage, dissociation of oligomers takes place, following in consequence reduction of molar volume. Formed monomers may create novel clusters or precipitate.
- 150 – 200 MPa - loss of tertiary structure. Polypeptides follow unfolding, dissociated the monomers aggregate or alternate their conformation. In this range of pressure molecular modifications depend on time and some of them can be reversed.
- 300 - 700 MPa - secondary structure failure. Protein rearrangements reach permanent denaturation, molecules cannot regain biological function. State depends on compression rate and on changes progression. [57, 58]

The possible routs of alterations in protein molecules during pressurization are illustrated in the figure 3. Red arrows reflect processes favored by pressure - they follow negative volume change. Mark of dashed arrow stands for the rather infrequent, in this case phenomena of aggregation. Regard to scheme, native proteins are likely to dissociate and unfold during compression. Molecules may regain their folded structure with pressure decrease. [2]

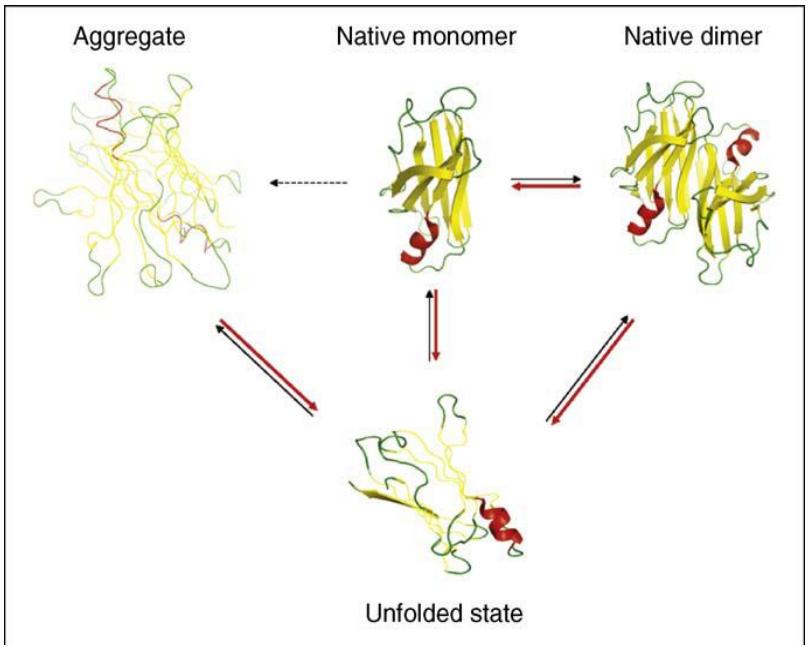


Figure 3 Impact of pressure on polypeptides, adapted from Aertsen, 2009 [2]

Worth of mention is the fact that the entire protein structure does not react equally to pressure, since it contains areas of variable compressibility. Thus, modifications of protein molecules are anisotropic. [59]

In the mechanism of protein denaturation two contrary processes are involved:

- reduction of cavities favored by decreasing volume;
- hydration.

The oligomers are conformationally stable if they can balance the breakage of energetically low bonds and prevent solvent penetration. Reduced size of cavities limits access of aqueous particles to invade inside polypeptides. However hydration which primarily affects external areas looses hydrogen interactions. As a consequence molecule gains flexibility and unfolding follows. Effect of internal hydration depends directly on the size of cavities. Solvent entering inside the structure is able to broaden the area of action - it reaches sites unavailable before. Thus, reactional surface is extended while protein undergoes unfolding. [49, 59]

In vivo, high pressure stress influences proteins to aggregate. Experiments of Mañas [56] proved that phenomena. Additionally, visualization with dyes showed favored peripheral arrangement of aggregates inside the microbial cells. With regard to results of

analysis, modifications of pressure values did not bring noticeable alterations in the general morphology of clumps. Interestingly, similar processes of aggregation were noticed in exponential- and stationary-phase cells. [56]

There are some indications stating proteins as a main target in pressure-inactivation of microorganisms. [49] Thus, special attention should be paid to the level of caused denaturation, since it is essential for the experiment proposed in this work to maintain cells viable.

1.3.4. How does HHP influence enzymatic activity?

Biological reactions in microbial cells may be modified by high pressure treatment due to:

- conformational changes in structure of enzymes - bringing increase or inhibition of their activity, or
- alterations in the mechanism of process - with regard to thermodynamics, for example to the Le Chatelier´s principle. [57, 58]

Reactions may be furthermore intensified with increased and facilitated contact between enzyme and substrate brought with membrane permeabilization. [58]

Thermodynamic principles govern also specificity of enzymes in high pressure conditions. Shifting the equilibrium of reaction, compression favors mechanisms involving substrates that give products occupying smaller volume. [2]

Molecules of enzymes are like other proteins, not-homogenous with parts of different ability to react on pressurization. Active sites are considered as areas that undergo the most significant changes in volume since they reflect highly compressible nature. Fluctuations can be described with two parameters: amplitude and frequency that are directly proportional - slight and quick alterations involving majority of structure or more significant but slower and limited to specific sites. Interestingly, binding of inhibitors may significantly change the compressibility of molecules. [59]

Available data shows that in mild pressurization, activity of enzymes is increased due to stabilization of the structure. This fact causes further improved thermal resistance of molecules. [2]

1.3.5. What is the effect of HHP on ribosomes?

Pressure has significant influence on ribosomes and synthesis of proteins is highly sensitive to its fluctuations. Treatments as low as of 40 - 60 MPa cause dissociation of ribosomal subunits. Those cellular structures are affected even more easily if present as free individuals than when gathered in polysomal forms. The sensitivity of ribosome on high pressure is determined by the small subunit - 30S, while the bigger one, 50S, does not contribute significantly. [58]

Pressurization above 70 MPa is said to be responsible for decreased rate of translation. Ribosomes are capable to reverse pressure-induced changes and switch protein synthesis to normal, suitable level. When cells are not able any more to recover the necessary number of ribosomes they inevitably die. [58, 60]

Presence of magnesium ions is crucial in stabilization of ribosomal structure. According to mentioned before hypothesis proposed by Gänzle [51], magnesium is lost due to the membrane permeabilization during pressurization. As a result, decreased concentration of Mg^{2+} causes destabilization of ribosomes and if this deficit lasts for a longer time, induces irreversible modifications. [58, 60]

Mechanism of protein synthesis inhibition induced by high pressure occurs at the early stage of the translation. Applied stress factor prevents formation of bond between mRNA and aminoacyl-tRNA in ribosome molecule. Interestingly, high pressure represses production of all polypeptides that are involved in transfer of amino acids on tRNA and consequently on mRNA complex. [58]

1.3.6. How does the HHP alter the nucleic acids?

Nucleic acids are said to be more resistant to high pressure treatment than proteins. This phenomenon is explicable since in stabilization of DNA structure hydrogen bonds are involved. Thus, pressurization is mostly reported to induce stabilization effect on those molecules. According to results presented by Sharma [61], pressure-stabilized plasmids demonstrate increased capability to enter inside the competent cells. [58, 61]

Several experiments on response of nucleoid for high pressure treatment demonstrated meaningful changes in distribution and thickness of the cell structure. Even moderate pressurization effects in condensation of the nucleoid which increases with pressure. The phenomenon is observed in exponential and stationary phase cells, but in

first ones appears more severe. Normally nucleoids occupy cytoplasm in a proportional manner but after high pressure stress they are located asymmetrically, sometimes cumulated in one location, close to membrane. [56]

As the hypothetical explanations of nucleoid condensation are suggested:

- direct increase of DNA density (without negative effect on biological function),
- indirect changes of protein-DNA complexes.

The latter may result from polypeptide denaturation depriving whole composite of activity. Possibly, it can be also associated with the damage of cell envelopes. Intracellular proteins require presence of divalent metal ions to maintain stable native structure. Since those ions are lost through interrupted membrane, polypeptides and their complexes lack their stabilizing activity. [49]

Additionally, pressurization affects the mechanisms of DNA replication and repair. For example, at 50 – 80 MPa gyrase loses the activity and as unable to conduct proper chain coiling inhibits the replication. Under pressure DNA susceptibility to activity of endonucleases increases. Thus, the enzymes may cut the molecule more easily. [58]

2. Materials and Methods

2.1. Microorganisms

The TOP 10 strain of *Escherichia coli* was purchased from Invitrogen. It was grown in the liquid LB medium at 37±1^{o}C in a shaking incubator at 160 rpm. The culture stock was stored at 4±1^{o}C on solid LA medium during the experimental work.

2.1.1. Electrocompetent cells

E. coli was prepared as electrocompetent according to electroporation protocol provided in the manual of Micro Pulser Electroporation Apparatus from Bio-Rad.

In the first step of procedure pre-inoculation was performed. Thus, 20 µL of cell suspension was added to 5mL of LB medium and incubated overnight at 37^{o}C, with shaking at 160 rpm. Next day, 180 mL of LB medium was inoculated with 1,8 mL of overnight culture and left in the incubator at 37^{o}C at 160 rpm. The growth of bacteria was monitored periodically by optical density measurements accomplished at an absorbance spectrophotometer. Since the cells from log-phase were recommended for obtaining the highest electroporation efficiency, the incubation was terminated when the absorbance of the suspension reached values of approximately 0,6 - 0,7 for 600 nm wave length (Varian Cary 50 UV-Vis Spectrophotometer). Immediately the culture was placed on ice and chilled for 20 minutes. All following handling was carried out at temperature close to 0 ^{o}C. The culture was equally divided and transferred to centrifuge tubes containing 30 mL each. The cells were pelleted by centrifugation. All centrifugation steps during the procedure were performed for 15 minutes at 4000 x g, at 4 ^{o}C. The supernatant was decanted and replaced with 30 mL of previously cooled 10% glycerol at each tube. The glycerol solution was kept close to 0 ^{o}C for all washings. The step was completed by centrifugation and subsequent discarding. The pellet was analogically resuspended and harvested two times more with respectively 15 mL and 1,2 mL of 10% glycerol. The precipitate was suspended in the final volume 1 mL of 10% glycerol and aliquots of 100 µL were prepared. *E. coli* cells were stored at -70±1 ^{o}C during all experimental work.

The average concentration of electrocompetent cells was calculated using spread plate technique and the value was (7,23±3,85)×10^{15} cfu/mL.

2.1.2. Non-electrocompetent cells

Non-electrocompetent cells were prepared following the protocol created for the experiment. 240 mL of LB medium was inoculated with 2,4 mL of fresh overnight culture and incubated at 37±1^{o}C at 300 rpm till cell suspension reached the optical density of OD_{600}≈0,6-0,7. The culture was chilled on ice for 20 minutes and proportionately distributed to 30 mL centrifuge tubes. Harvesting step was performed for 10 minutes at 500 rpm, at 4 ^{o}C. After discarding, the pellet was resuspended in 2 mL ice-cold 10% glycerol and the aliquots of 100 µL and 400 µL were prepared. Samples were deposited at -70±1^{o}C for all experimental work.

Determined with spread plate method the average concentration of cell suspension was equal to (4,14±0,99)×10^{9} cfu/mL.

2.2. Plasmid

2.2.1. Commercial pUC19

The commercial sample of plasmid pUC19 was provided by Invitrogen. The plasmid of concentration equal to 10 pg/µL was stored in the TE buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, at -24±1^{o}C.

2.2.2. Extracted pUC19

The extractions were carried out from culture of *Escherichia coli* TOP10 transformed with commercial pUC19 by electroporation. The extraction was performed applying two distinct kits: Qiagen Plasmid Mini Kit and GeneJET Plasmid Miniprep Kit (Fermentas). Only the latter one provided satisfactory concentration and acceptable purity of plasmid.

The concentration of pUC19 utilized in experimental work was adjusted to 100 ng/µL by diluting the plasmid sample with Elution buffer provided with GeneJET Plasmid Miniprep Kit (Fermentas).

2.2.3. Quantification of extracted plasmid

- Agarose gel electrophoresis

The presence and quality of extracted plasmid was confirmed by agarose gel electrophoresis. The analysis was performed loading 2 µL (after electroporation) and 10

µL (after pressurization) of samples on the 1% agarose gel (SeaKem LE Agarose, Lonza) and the run lasted 80 minutes at the voltage of 80 V.

- Absorbance measurements

The concentration of the extracted plasmid was determined with Nano Drop 1000 Spectrophotometer purchased from Thermo Scientific. The absorbance measurements were carried out at 260 nm of wave length. Recorded values of ratios A_{260}/A_{280} and A_{260}/A_{230} indicated efficiency of purification.

- Control electroporation

The transformation competence of extracted plasmid was confirmed with control electroporation. The test was performed in accordance to standard electroporation protocol (see 2.3.1. Electroporation).

2.3. Genetic transformation

2.3.1. Electroporation

The electroporation was accomplished applying MicroPulser Electroporation Apparatus from Bio-Rad (Serial Number: 411BR 3991).

The procedure of transformation was performed according to the protocol set forth in the manual provided with equipment. As it recommends, the MicroPulser was adjusted to Ec1 program which is suggested for electroporation of *Escherichia coli* in 0,1 cm cuvettes. Regard to the set parameters, the pulse was identified with 1,8 kV of applied voltage and 18 kV/cm of generated electric field strength. The time constant was referred to reach approximately 5 msec, although precise value had to be confirmed after respective transformation.

The transformation procedure was conducted in sterile conditions in the laminar flow chamber. Firstly the sample of electrocompetent cells, 1,5 mL Eppendorf tube, 0,1 cm cuvette and chamber slide were placed on ice. When bacteria suspension and plasmid were both completely thawed, 100 µL of *E. coli* and 2 µL of pUC19 were pipetted to a tube and mixed moderately. After one minute incubation on ice the mixture was transferred to ice-cold cuvette, placed in the slide and driven to the chamber. The pulse was induced on the

formerly arranged and ready to use electroporator. Immediately, the cell suspension was mixed with 1 mL of SOC medium utilizing disposable sterile Pasteur pipette provided with cuvette. This step which is essential for bacteria recovery and consequently for transformation efficiency was carried out rather promptly. The content of cuvette was shifted to 1,5 mL Eppendorf tube and incubated for 1 hour at 37±1^{o}C at 160 rpm. The pulse characteristics: voltage and time constant were recalled and recorded for each transformation.

2.3.2. Innovative methodology

The transformation using an innovative methodology was performed according to the protocol created during this study. The procedure started by thawing on ice 400 µL samples of non-electrocompetent cells . When ready, 4 µL of unfrozen homogenized plasmid were joined to them and mixed smoothly. The suspension was transferred to 400 µL Eppendorf tubes utilizing the glass Pasteur pipette. Attention was paid to avoid air bubbles inside the tubes. The tops of tubes were sealed with sterilized parafilm, placed in the separate bags and submerged in ice. The Styrofoam box with ice and cooled samples was carefully transported to the laboratory where the pressurizing equipment is. Directly before each experiment plastic bags were closed with heat utilizing vacuum sealer.

The treatments with high hydrostatic pressure were realized in the High Pressure U33 equipment provided by Institute of High Pressure Physics in Poland. Samples prepared for individual operation were placed in the vessel of 100 cm^3 containing as a pressure transmitting medium propylene glycol and water in the volumetric ratio 1:1.

After completing pressurization samples were chilled on ice and transported back to the Department of Biology. All the volume was pipetted to the 2 mL Eppendorf tubes filled with 1,5 mL of SOC medium and incubated for 1 hour at 37±1^{o}C at 160 rpm.

2.4. Methods of quantification

2.4.1. Quantification of microorganisms

The concentration of cell suspension was determined with the spread plate method.

The plates of LA medium were prepared in sterile conditions, in laminar flow chamber. The medium at approximately 50^{o}C was distributed on dishes (15 mL) and let to solidify close to flame. Random samples were diluted with 0,9% NaCl and mixed.

Obtained in this manner variously concentrated solutions were plated on LA medium by uniform spreading 100 µL of respective dilutions operating with sterile Drigalski spatula. After 24 hour incubation at 37°C Petri dishes with number of 30 to 300 of grown colonies per single plate were identified as adequate to quantification. Obtained counts and dilutions served for subsequent determination of number of cells forming units per milliliter.

2.4.2. Quantification of transformants

The number of transformants was determined with spread plate method applying selective medium. Presence of antibiotic inhibits growth of cells that did not incorporate the plasmid.

The plates were prepared in the laminar flow chamber retaining all the asepsis rules. To autoclaved 250 mL of LA medium at approximately 40°C was pipetted 125 µL of Ampicillin (stock solution of 50 mg/mL) to obtain its final concentration equal to 50 µg/mL. The medium was gently mixed to reach uniform distribution of antibiotic and to avoid bubbling. Dishes were identified and filled with approximately 15 mL of medium each and left to solidify.

Since the viable bacteria observed primarily on Petri dish had formed small and irregular colonies, the final concentration of Ampicillin in the LA medium was raised to 100 µg/mL. Thus, analogous appropriate volumes were pipetted. The new concentration of the antibiotic was maintained until the end of experimental work.

Petri dishes were prepared by pipeting respectively 40 µL and 80 µL of cell sample and spreading them with the sterilized Drigalski spatula on the medium surface. Plates were incubated for 24 (after electroporation and pressurization) and 48 (exclusively after pressurization) hours at 37°C and colonies forming units were counted providing total number of colonies required to determine transformation efficiency.

2.4.3. Efficiency of transformation

With regard to the manual of MicroPulser Electroporation Apparatus the transformation efficiency (E_T) was calculated from the equation:

$$E_t = \frac{Total\ number\ of\ colonies\ grown\ on\ plate\ [transformants]}{Amount\ of\ plated\ DNA\ [\mu g]}$$

The value of general transformation efficiency achieved with procedure recommended in the electroporator guidebook is said to be 10^9-10^{10} transformants/µg of DNA.

2.5. Discrimination between transformants and contaminants

48-hour incubation may entail hazard of growth of non-transformants. Additional verification of colonies formed on selective Petri dish was required.

2.5.1. Expression of β-galactosidase

In order to confirm bacteria transformation selective Petri dishes permitting distinction of blue and white colonies were prepared. The surface of solid medium composed of LA and Ampicillin was covered with a layer of equal volumes (40 µL) of X-gal and IPTG. Cells were grown on the plates for 24 hours at 37±1^{o}C.

2.5.2. Microscopic observation

The microscopic observations were performed utilizing Nicon Eclipse 80i Microscope. Cell samples were placed on slides (VWR ECN 631-1550) and viewed under the objective of resolution 100 X with immersion oil.

2.5.3. Plasmid extraction

The presence of plasmid in grown cells was attempted to be confirmed by its extraction. Plasmid Miniprep Kit II purchased from E.Z.N.A. and GeneJET Plasmid Miniprep Kit from Fermentas were utilized according to supplied protocols. Some attempts were made introducing additional pre-incubation step for 10 minutes at 37±1^{o}C with lysozyme (Eurobio) preceding standard procedures.

The trial to confirm presence of the plasmid utilizing Genomic DNA Purification Kit provided by Fermentas was also undertaken. The procedure was performed according to the economic protocol provided by MicroLab of University of Aveiro. As previously, the breakage of cells did not occur.

2.5.4. Gram staining

The microscopic examination of bacteria is difficult because the refractive index of cell content and of membrane has almost the same value. Thus, usually in order to improve

observation, staining techniques are employed. Gram method permits distinction between two groups of bacteria: Gram-positive and Gram-negative.

To perform Gram staining singly colonies were picked and uniformly spread in water drop on surface of slide. Water was evaporated above the flame and in order to make cells adhere to the glass. Then the sequence of washings with crystal violet, iodine solution, decolorizing reagent and safranin was accomplished. Each washing step lasted 1 minute and was followed by cleaning with water stream. The slides were left to dry and when ready they were subjected to microscopic observation (Nicon Eclipse 80i Microscope) with immersion oil.

2.6. Culture media and asseptic methods

2.6.1. LB and LA medium

LB broth (MILLER) for liquid cultures was purchased from Merck. Typically the medium is composed of Peptone from casein (10,0 g/L), Yeast extract (5,0 g/L) and Sodium chloride (10,0 g/L).

LA medium was prepared from LB broth (25 g/L) and agar for microbiology use (15 g/L), both supplied by the Merck.

2.6.2. SOC broth

Rich in nutrients SOC medium was prepared in accordance to standard protocols. The content of broth is presented in the table 4. Particular components were dissolved in distilled water.

Table 4 The content of a rich SOC broth.

Components	Supplier	Content per L
Tryptone casein peptone	Amresco	20,0 g
Yeast Extract	Alfa Aesar	5,0 g
Sodium Chloride	Merck	0,5 g
Potassium Chloride	Merck	187,0 g
D(+)-Glucose monohydrate	Merck	3,6 g

2.6.3. The asepsis

Due to the high risk of microbial contamination of bacteria culture of interest, it is important to maintain sterility during the all operation steps and handling. Hence, all

material utilized in experiment, including glass and plastic equipment, solutions and broths were preferably autoclaved for 20 minutes at 121^{o}C. Metal tools (like loops and forceps) were sterilized in flame or by submersion in ethanol.

Material employed at work in high hydrostatic pressure conditions as non-autoclavable was exposed for 15 minutes to ultraviolet radiation inside the laminar flow chamber.

Samples were prepared and preferably handled in the laminar flow chamber wiped previously with ethanol and operated close to flame. Each manipulation was preceded by sterilization of hands with ethanol.

3. Results and Discussion

3.1. Transformation with conventional methodology

The work began with determination of electroporation efficiency for chosen host-plasmid pair. Prepared electrocompetent cells of *E. coli* TOP10 were subjected to transformation by electric pulse and the procedure was evaluated according to the manual. Obtained values were lower than referred in the protocol rates of general efficiency (10^9 – 10^{10} transformants/µg of DNA) reaching the average values of $(9{,}47\pm2{,}00)\times10^7$ and $(6{,}30\pm0{,}83)\times10^7$ transformants/µg of DNA for procedure with plasmid concentration of 0,01 µg/mL and $(1{,}18\pm0{,}37)\times10^7$ and $(3{,}44\pm0{,}56)\times10^7$ with plasmid concentration of 100 µg/mL (see table 5, Appendices, p. 60). Since rates obtained with commercial and prepared samples of pUC19 were in the same order of magnitude, the quality of extracted plasmid was considered to be satisfactory.

The electroporation of non-electrocompetent cells was not achieved. The reason of failure was excessive conductivity of the cell suspension. That in turn was caused by high content of salts in the solution due to insufficient washing during preparation of cells.

3.2. Transformation with an innovative methodology

3.2.1. Optimization of treatment time

The initial objective of the experimental work was to establish the range of stress factor that causes moderate reduction of cells. That would ensure that applied pressurization affects the bacteria but viability is maintained on reasonable level permitting detection of possible transformants.

The experimental work started with treatments under 100, 200, 300 and 400 MPa and each pressurization lasted 5 minutes. Those conditions were chosen after being consulted with the results presented by Gänzle [51]. The viability of bacteria was determined by cultivation on solid media. Since on plates with nonselective medium prepared 5 days after experiment no cell growth was observed, it was supposed that applied conditions caused too severe damage to microorganisms. Admitting that there were a few survivors after treatment, they must have loose viability during the storage at 4^oC.

Plating on Petri dish with selective medium did not result in formation of colonies. This observation was not surprising in face of the existence of no survivors in the

nonselective plates, indicating that the pressure treatment had to be optimized, in order to cause less damage on cells.

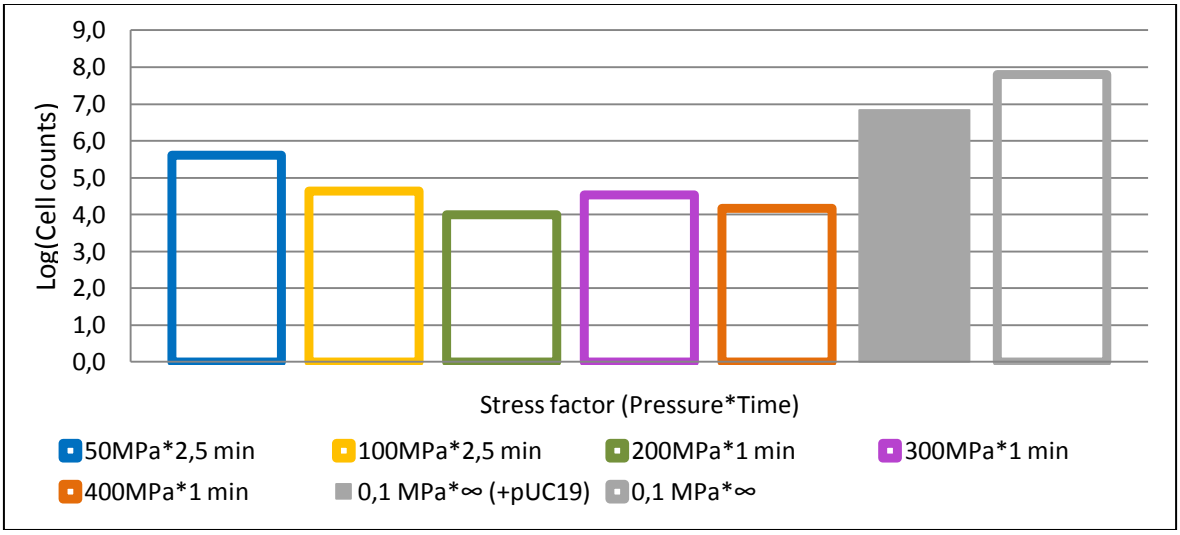
Taking into account recorded high inactivation level, it was suggested to perform the experiment applying milder conditions. A single factor was selected to be changed in order to assess objectively its influence on the cell viability. It was decided to perform a trial with reduced pressurization times.

3.2.2. Optimization of pressure value

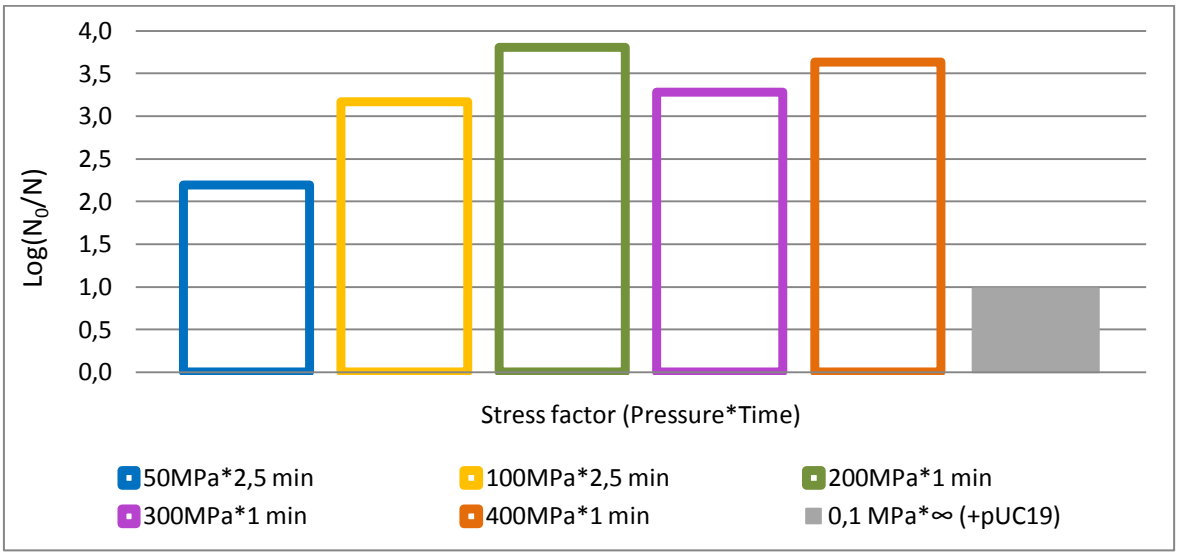
Since the conditions were recognized as causing too drastic inactivation of bacteria, the treatment time was decreased. Thus, pressures 50 and 100 MPa were applied during 2,5 minutes and 200, 300 and 400 MPa during 1 minute. Blanks, not subjected to high pressure, were conducted on double samples: one containing only cell suspension and the other, cells mixed with plasmid. Afterwards, specimens with DNA were plated on selective Petri dishes giving indication about occurrence of transformation.

The results of cell counting on LA plates are demonstrated in the table 6 (Appendices, p. 61). Presented values were obtained after 5-day long storage at 4^{o}C. The blanks were described as subjected to normal pressure of 0,1 MPa during undefined time.

For the analysis of experimental results the term “stress factor” was defined. The created term encompasses all treatment conditions that contribute to final effect on the viable cells, here: pressure and time. On the graphs 1 and 2 filled bars stand for samples to which plasmid was added.



Graph 1 Results of cell viability after treatment depending on stress factor (Pressure*Time).



Graph 2 Decimal reduction of cells caused by treatment depending on stress factor (Pressure*Time).

Graphs 1 and 2 present how the applied treatments affected the viability of *E. coli* TOP10. Regard to them, the aim of assay – generation of more moderate stress on bacteria was obtained since survivors succeed to grow on Petri dishes and gave reliable countings. The lowest rate of lethal effect was obtained with pressures 50 and 100 MPa. Thus, that range of pressurization values should be maintained during following experiments. However, since the desired viability rate was defined as approximately 80-90% of the initial value, the stress factor was recognized as still requiring to be softened.

The assay provided only single data indicating the influence of DNA on bacteria viability. According to it, presence of plasmid reduced the number of colony-forming units by one order of magnitude. To verify what is the impact of presence of pUC19 on *E. coli*, double specimens should be subjected to treatments: one containing only cell suspension and the other – enriched in plasmid, and afterwards both types of samples ought to be grown on nonselective medium, determining the viability.

Since no colonies of *E. coli* were observed on selective plates, there were no indications that transformation had occurred. One of the factors influencing the efficiency of transformation is the concentration of the DNA . With regard to the manual of electropulser, increase of DNA concentration results in improved frequency of transformation (transformants/survivors) since it causes higher probability of successful

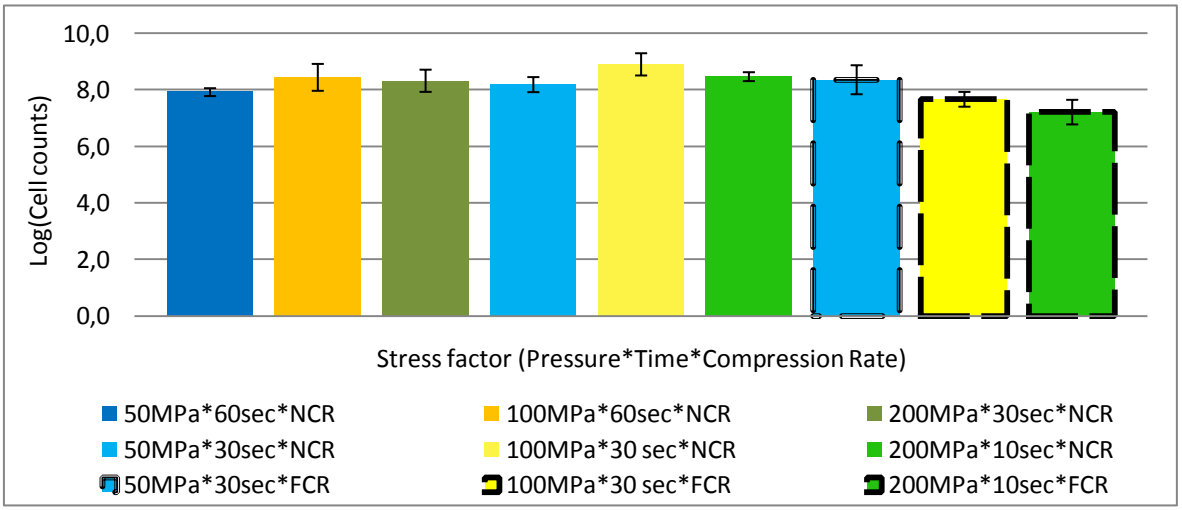
plasmid delivery to cell. Hence, application of increased amount of pUC19 could possibly bring successful improvement to new methodology.

3.2.3. Optimization of inactivation level

The objective of the assay was to determine the synergistic and individual effect of several factors contributing to treatment on the cell inactivation. Thus, samples containing exclusively bacteria suspension were subjected to high pressures. The experiment was performed under three different pressures: 50, 100 and 200 MPa and the collected results are presented in table 7 (Appendices, p. 62). To facilitate test analysis, particular factors are subsequently discussed below. On the graphs, bars without contour state for treatment at normal compression rate (NCR, approximately 5 MPa/sec) and those with dotted line – for fast compression rate (FCR, approximately 10 MPa/sec).

- Impact of stress factor

In the performed experiment the stress factor was defined as combined effect of: pressure, time of treatment and compression rate. According to visualized on the graph 3 results, in general, the fluctuations of the cell inactivation caused by applied stress factors varied only moderately between each other. Thus, the impact of tested variables on bacteria viability was comparable and approximate. However, as the treatments causing the less severe damage to cells, were recognized the series with the pressurization times of 60, 60, 30 seconds at respectively 50, 100 and 200 MPa at normal compression rate.



Graph 3 Results of cell viability after treatment depending on stress factor (Pressure*Time*Compression rate).

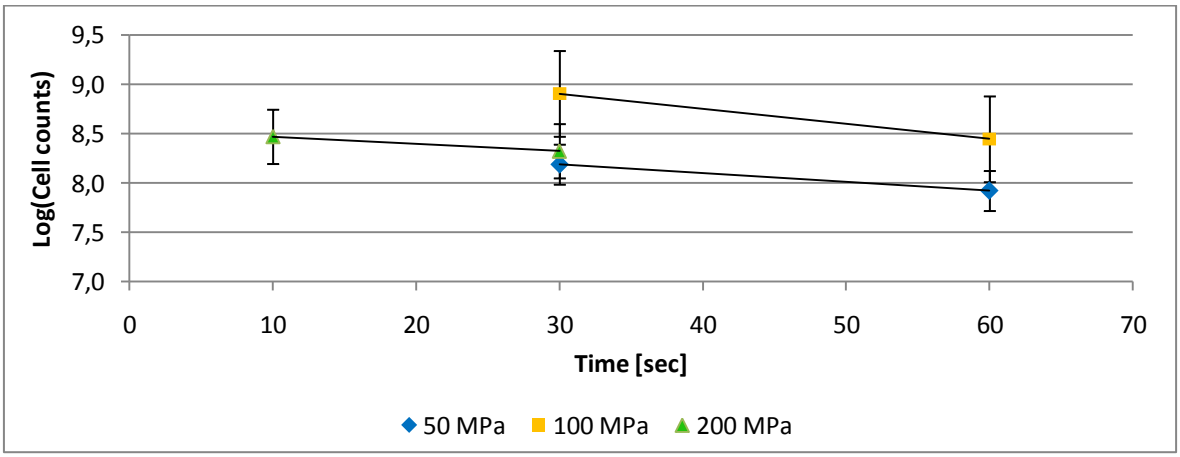
The compression rate was recognized as the variable having - among tested factors - the most meaningful impact on the cell inactivation. Increased compression rate caused more radical reduction of number of survivors than regularly applied one (NCR). It is understandable since cells can adapt easier to changes occurring less drastically. Biological systems need to adjust to altered conditions and reach the new equilibrium state.

Between arrays there was observed a tendency - the number of colony-forming units depending on applied treatment was preferably distributed in accordance with the Gaussian curve. Thus, the highest number of survivors was reflected by bars corresponding to tests realized under 100 MPa. It could be concluded that this pressure caused less severe damage to cells than two others tested. *E. coli* TOP10 seemed to be more sensitive to 50 and 200 MPa.

Unfortunately, because of inappropriate dilutions, cell counts for blank samples were not obtained. Hence, conclusions regarding the reduction rate relative to initial bacteria population could not be achieved.

- Impact of pressurization time

The relation between cell viability and treatment time is presented on graph 4.

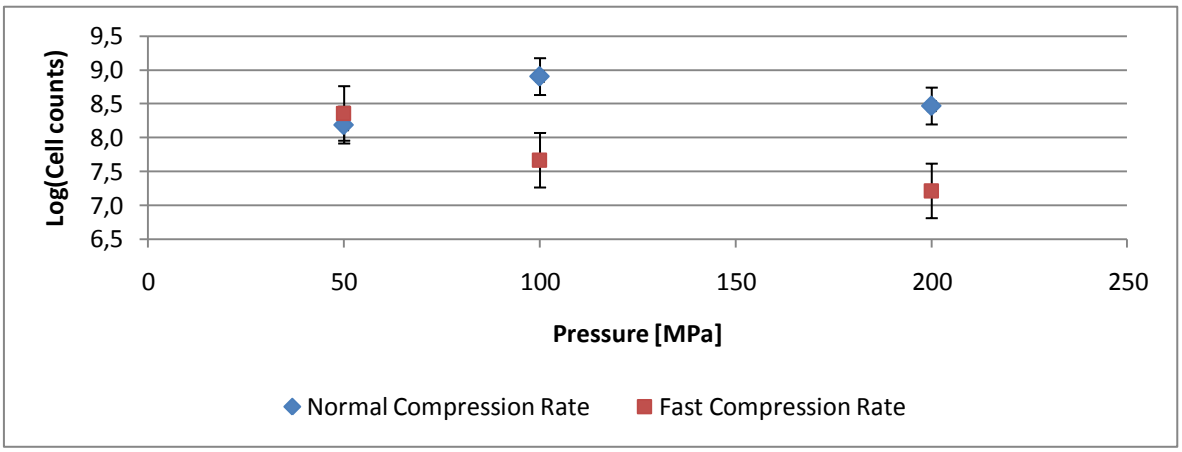


Graph 4 Results of cell viability depending on treatment time. Data for normal compression rate.

The longer pressurization was performed the higher inactivation effect was observed. Slopes of lines reflecting 50, 100 and 200 MPa are very similar - the change of number of survivors in time was almost the same. It may be concluded that those pressures had similar inactivation effect on *E. coli* at the analyzed range of time. Interestingly, the

difference in values for 50 and 200 MPa after 30-second treatment was slight while pressurization at 100 MPa during the same time resulted in higher cell counts.

- Impact of compression rate

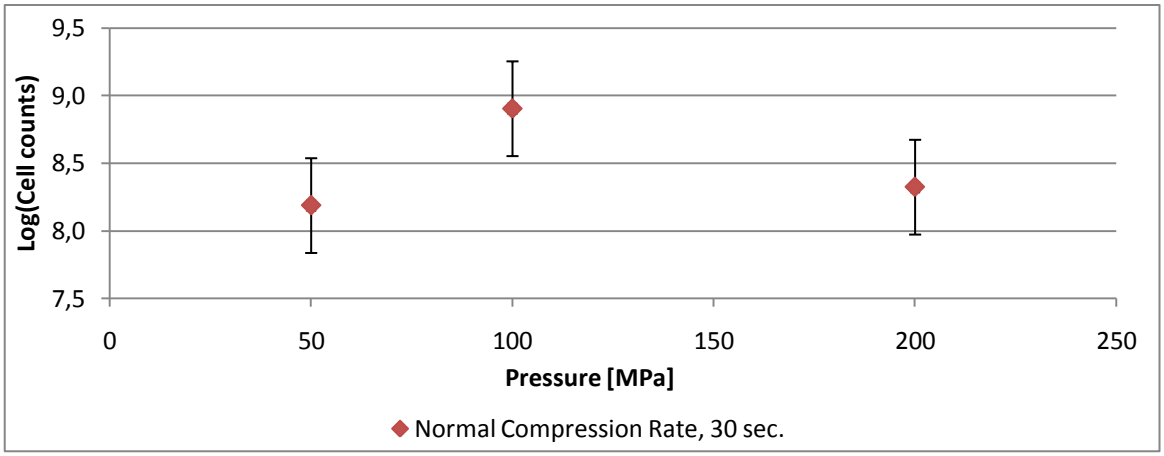


Graph 5 Comparison of influence of compression rate on cell viability after treatment depending on pressure. Data for treatments at 50, 100 and 200 MPa during respectively 30, 30 and 10 seconds.

As concluded before and assured on graph 5, high compression rate seemed to have more lethal effect on cells than standard one. During pressurization, compensation of changes occurring slower might be realized more effectively. Interestingly, for lower pressures (50 MPa) the compression rate appeared as not having as significant impact on cell viability as for higher (100 and 200 MPa).

- Impact of pressure

Graph 6 confirms already mentioned tendency to the distribution of Gauss. Regard to the graph, pressure of 100 MPa resulted in the highest viability of cells, while reduction observed under 50 and 200 MPa did not vary significantly between each other.

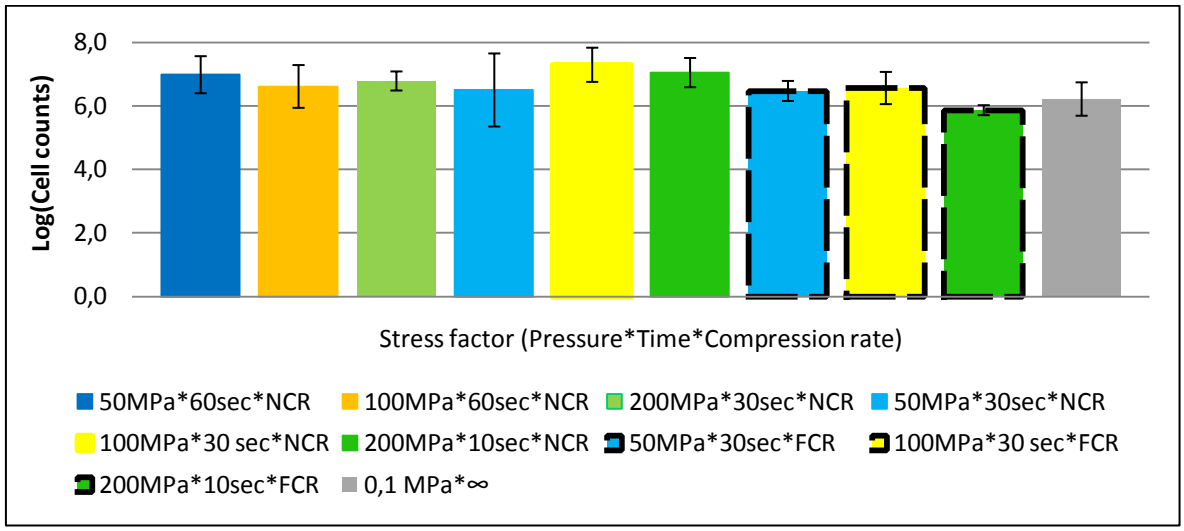


Graph 6 Results of cell viability after treatment depending on pressure. Data for pressurizations at normal compression rate (approximately 5 MPa/sec.) during 30 seconds.

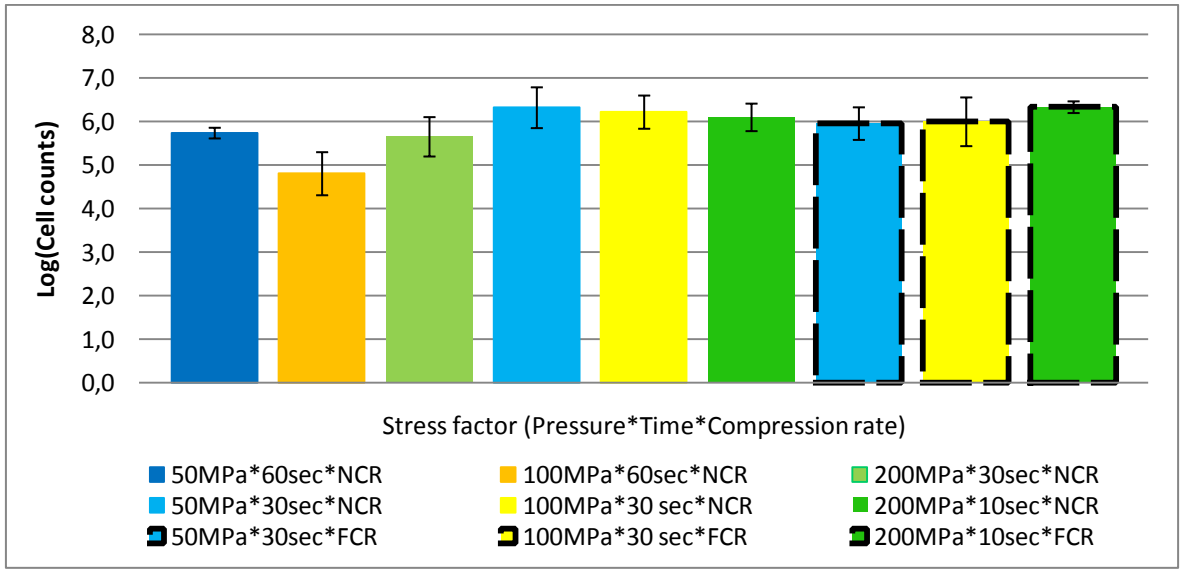
- Addition of SOC broth

One of the objectives of the experiment was to define the importance of the time interval between performed treatment and addition of nutritionally rich medium. Thus, two different routes were compared: with delayed (approximately 10 minutes) and immediate supplementation with SOC.

The results of plating for both routes are presented at the table 7 (Appendices, p. 62). Comparable data was collected 3 days after the experiment – inappropriate dilutions prevented availability of results after the first counts. Graphs 7 and 8 visualize viability of cells obtained after delayed and immediate addition of SOC. To facilitate comparison, bars of the same appearance stand for equal stress factor at both graphs.



Graph 7 Results of cell viability after treatment depending on stress factor (Pressure*Time*Compression rate). Data for samples with SOC supplemented after delay (app. 10 minutes).



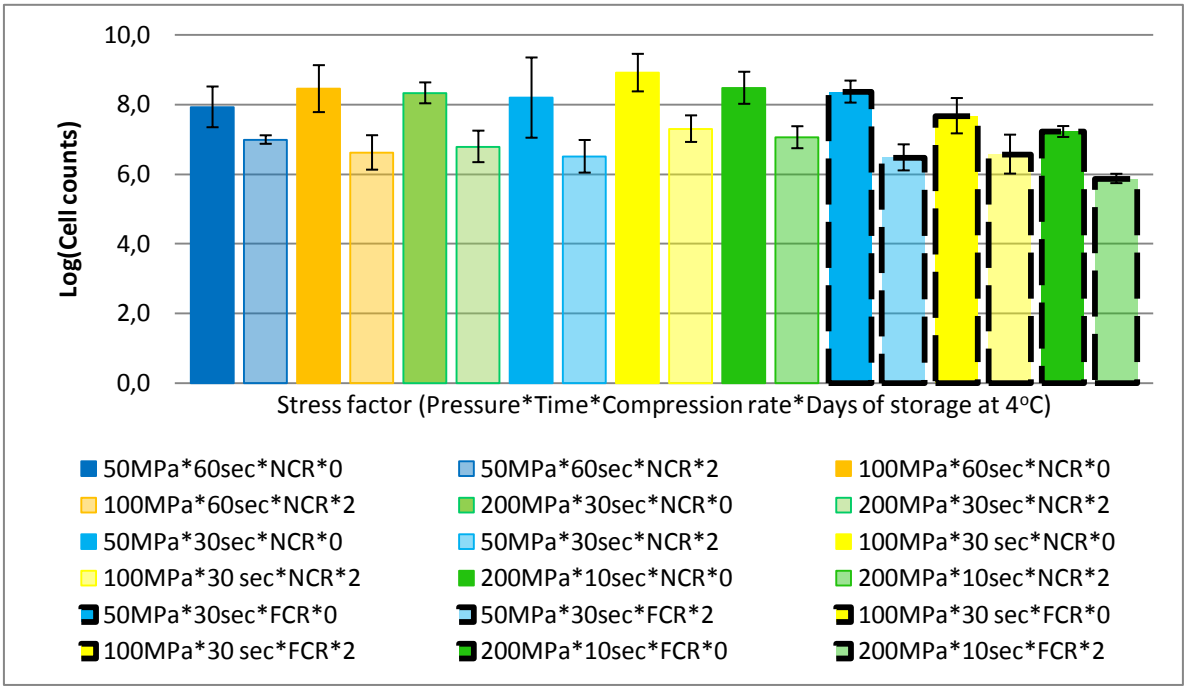
Graph 8 Results of cell viability after treatment depending on stress factor (Pressure*Time*Compression rate). Data for samples with SOC supplemented immediately.

According to the obtained results, number of survivors of relevant stress factors was noticed to be on average about one order of magnitude higher for delayed addition of SOC. It may be concluded that *E coli* need time to recover pressurization. Too quick supplementation in nutritionally rich medium can be considered by living systems as another stress factor to face with. They are capable to deal with it only if they regain primarily disturbed balance.

With regard to a conclusion, it was decided to maintain the layout of standard protocol - all subsequent experiments were performed with delayed addition of the SOC medium.

- Inactivation during the storage

The counts of cells forming units on Petri dishes were performed 1 and 3 days after experiment. Collected data permitted comparison of the bacteria inactivation during the storage at 4°C for results corresponding to the samples that followed standard protocol (delayed addition of SOC).



Graph 9 Inactivation of treated cells during the 0- and 2-day storage at 4°C depending on applied stress factor (Pressure*Time*Compression rate*Days of storage at 4°C).

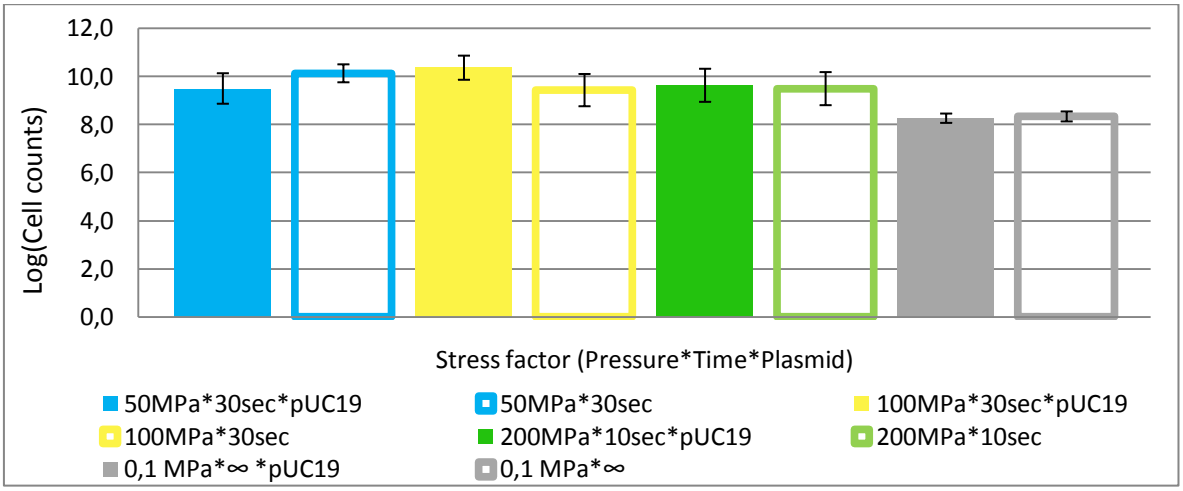
The results are illustrated on the graph 9. Opaque bars state for 0-day storage at 4°C and transparent ones – for 2-day storage. It can be recognized that stored samples resulted in the reduction of cell viability of 1,5 order of magnitude in average. This observation confirms the importance of preparation of adequate dilutions of samples that are plated after experiment.

3.2.4. Evaluation of increased concentration of plasmid

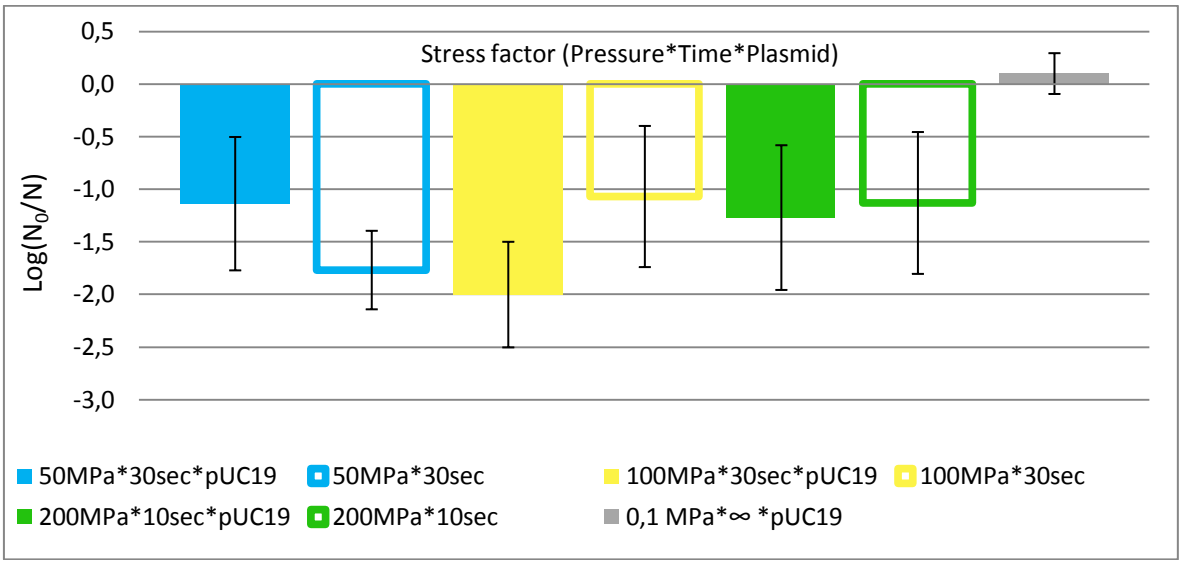
After accomplishing DNA extraction and quantification, the concentration of plasmid was adjusted to 100 ng/µL. Duplicates of samples were subjected to each pressure: one containing exclusively cell suspension and the other enriched in 2 µL of pUC19. After applied treatment both were plated on non-selective medium to determine the influence of plasmid on bacterial viability.

According to conclusions reached at previous assay, conditions giving the lowest reduction rates were maintained. Thus, the experiment was performed under 50, 100 and 200 MPa during respectively 10, 10 and 30 seconds. All pressurizations were accomplished at normal compression rate (approximately 5 MPa/sec). The results are shown in table 8 (Appendices, p. 63).

Figures 10 and 11 visualize respectively viability and decimal reduction of bacteria after each treatment. Bars filled with color stand for samples to which pUC19 was added and contoured reflect those without plasmid. Since the experiment analyzed also impact of plasmid, its presence/absence was included in definition of stress factor.



Graph 10 Results of cell viability after treatment depending on stress factor (Pressure*Time*Plasmid).



Graph 11 Decimal reduction of cells caused by treatment depending on stress factor (Pressure*Time*Plasmid).

Obtained values related to blanks were found in satisfying range, confirming the accuracy of applied stress factors. The results acquired for duplicates did not vary significantly. Those fluctuations could be caused by variation of initial cell concentration and as such were recognized. Concluding, no indications were found that presence of plasmid affect the viability of bacteria.

Interestingly, the untreated samples resulted in lower cell viability than pressurized ones. Negative values of decimal reduction on graph 11 correspond to an increase in number of cell forming units. It could indicate activating effect of pressure on microorganisms or on their spores (not applicable to *E. coli*). [62] Possibly, it might be the result of disintegrating effect of high pressure on agglomerates and clusters. [49] Finally, the most undesirable observation can be the effect of contamination. Samples which were not subjected to pressurization did not require to be transferred to 400 µL Eppendorf tubes. Since in handling of blanks one operational step was eliminated, they face with reduced hazard of loss of asepsis. Concluding, as a recommendation for future experiments, double blanks should be prepared: with and without the change of tube. In this way, the influence of transfer step in handling procedure would be determined.

There was no indication of colony formation on selective Petri dishes after 24-hour incubation at 37^{o}C. However, it was taken into consideration that pressurized cells could differ in growth requirements from standard population. Thus, the time of incubation was

prolonged to 48 hours and afterwards plates were observed. Figures 4 - 7 present achieved results.

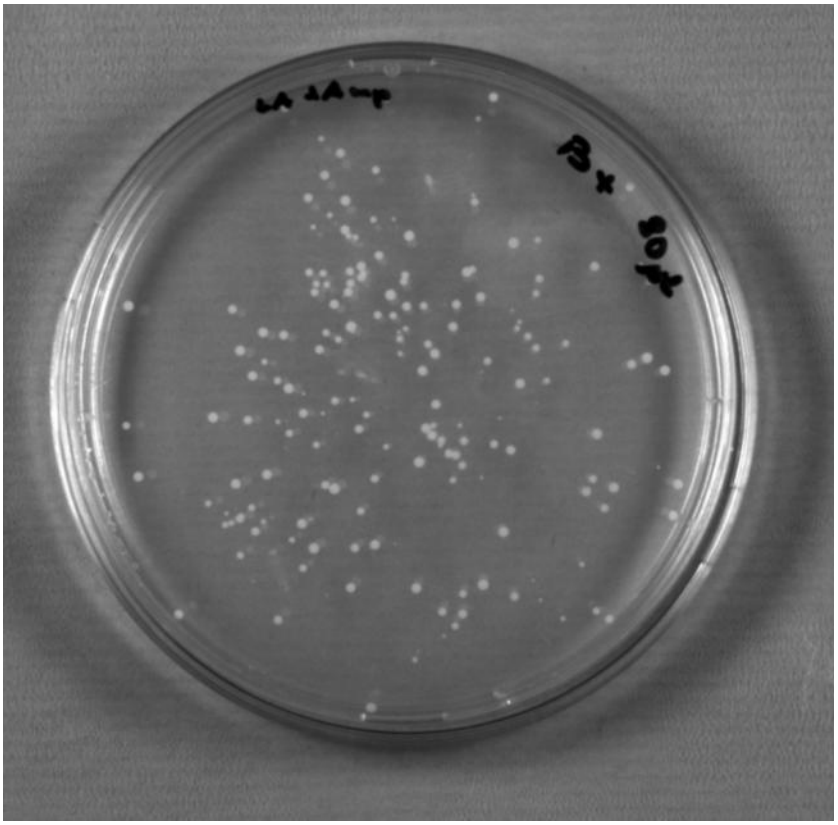


Figure 4 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Blank i.e. not pressurized sample of *E. coli* and pUC19 (0,1 MPa, ∞); Volume plated: 80µL.

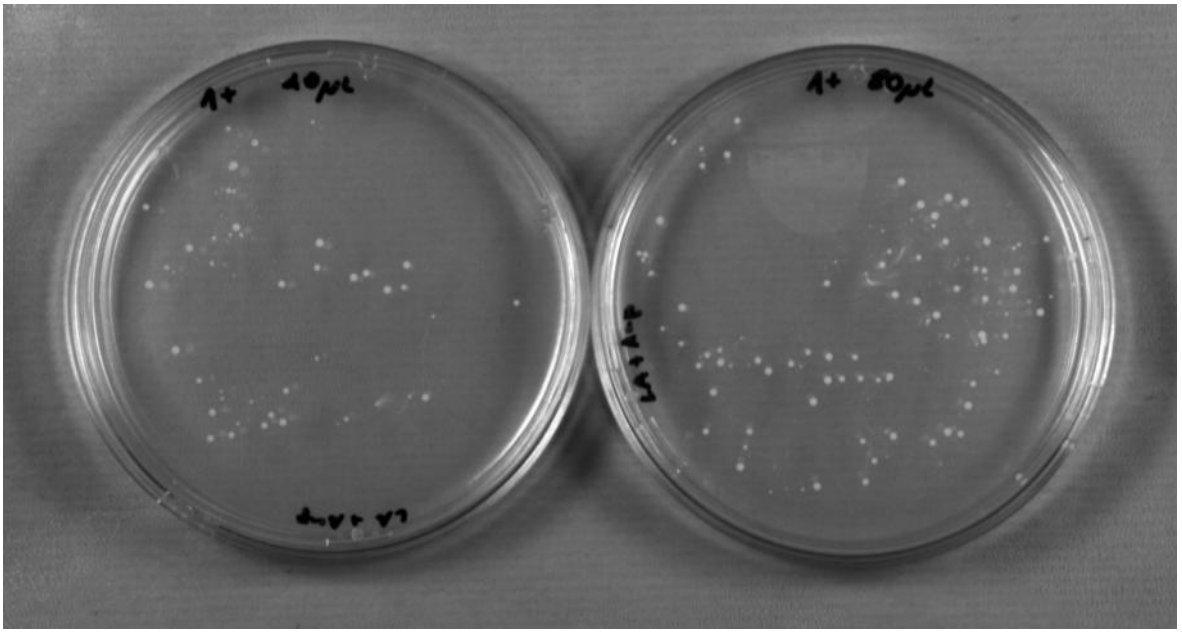


Figure 5 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 50±5 MPa, 30 sec.; Volume plated: 40 µL (left plate) and 80 µL (right plate).

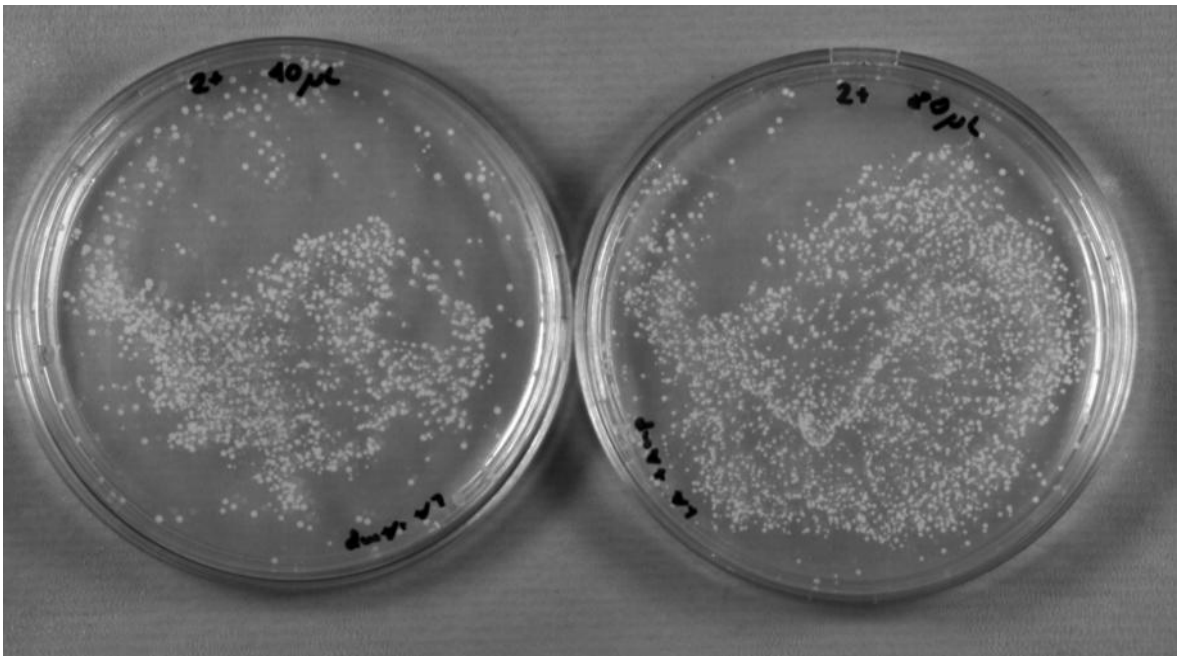


Figure 6 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 100±5 MPa, 30 sec.; Volume plated: 40 µL (left plate) and 80 µL (right plate).

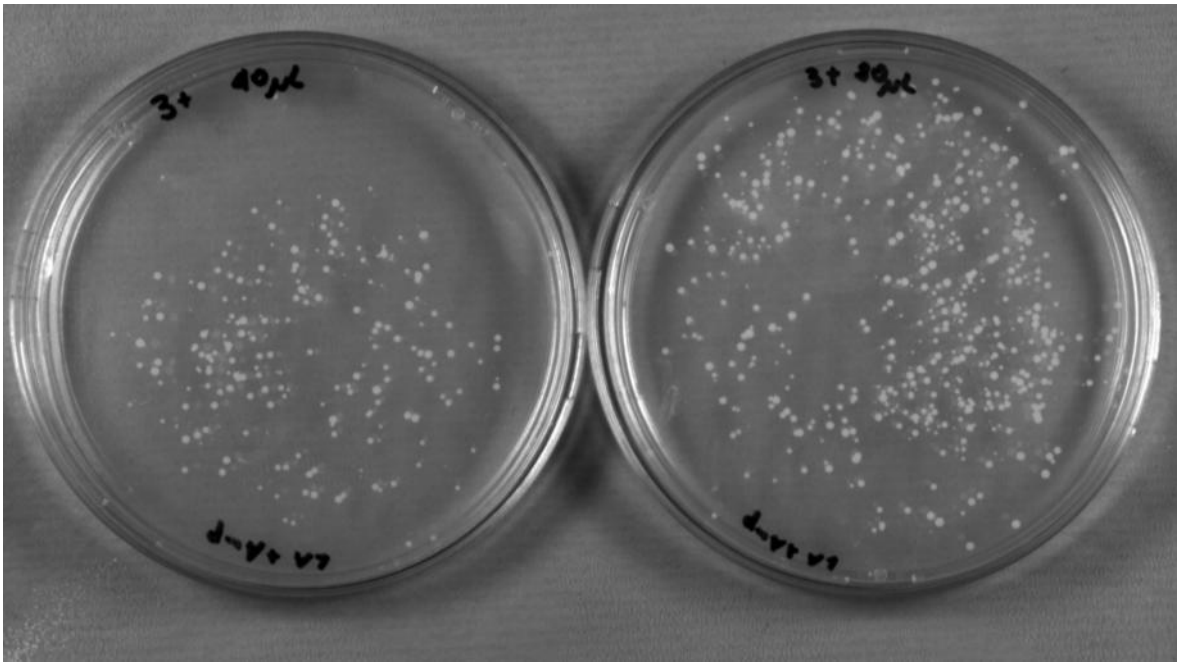


Figure 7 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 200±5 MPa, 10 sec.; Volume plated: 40 µL (left plate) and 80 µL (right plate).

48-hour long incubation resulted in a cell growth on selective plates. Since observed colonies must represent ampicillin resistant microorganisms, they were considered as possible successful transformants. According to the presented photos, there was notable correlation between number of colony-forming units and applied treatment. For example, density of cell growth after pressurization at 100 MPa was recognized as the highest (figure 6). Relating this observation to previously reached conclusion (viability of cells treated under 100 MPa was the highest in tested range) could be concluded that microorganisms found on selective dishes should be *E. coli* TOP10. Moreover, number of colony-forming units corresponded to volume plated what agreed with other indications of presence of transformed cells.

During visual analysis of plates, formed colonies were found heterogeneous. Some of growing individuals were recognized as filamentous fungi. However, it was not surprising observation because prolonged time of incubation gives opportunity to grow organisms that do not find created conditions as optimal. Fungi being non-sensitive to ampicillin and requiring longer incubation than bacteria could appear and successfully colonize the plates. Moreover, they are spores-forming organisms thus those highly resistant forms are probable to survive all the treatments and germinate on the plates.

To confirm the origin of colonies growth on selective Petri dishes, the extraction of plasmid was undertaken. The attempt was performed utilizing E.Z.N.A. Plasmid Miniprep II Kit and GeneJet Plasmid Miniprep Kit but none of them succeeded. Observations during extraction procedures indicated failure of lysis step in both methods – the lysate did not become viscous and clear as expected. Agarose gel electrophoresis confirmed conclusion because after visualization no DNA band was found on the spectrum.

Possible explanation for the occurred difficulties with breaking cells could be membrane modification due to the pressure treatment. It is known that barophilic organisms are characterized with more rigid cell envelopes. Pressurization is reported as a factor causing lost of structural fluidity and flexibility by membranes. [53] However, there are microorganisms naturally more resistant and so more difficult to lyse than *E. coli* is.

In order to determine the presence of the plasmid in grown individuals, blue/white screening method was realized. Figure 8 presents result of the performed plating on selective Petri dishes enriched with X-Gal and IPTG (see 1.1.4. The vector – why pUC19?).

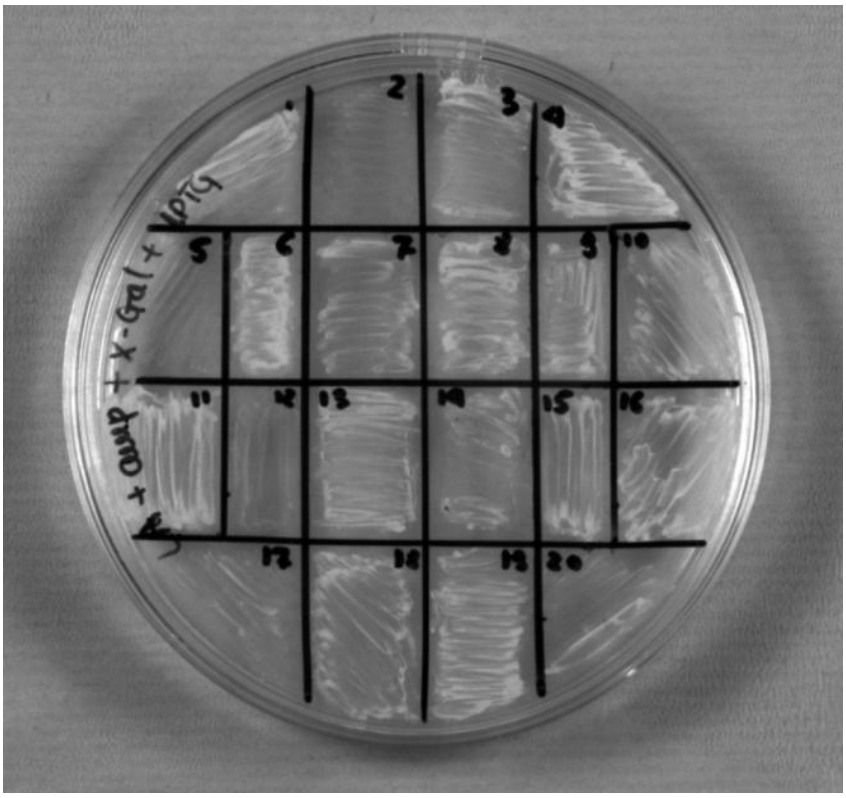


Figure 8 Results of blue/white screening of 20 morphologically diverse colonies. Petri dish: LA, ampicillin, X-Gal and IPTG; Incubation: 24 hours, 37ºC.

As visualized on figure 8 only white colonies grew on the plate indicating that none of individuals express the β-galactosidase that metabolizes X-Gal. However, it could not be concluded that transformation did not occur. It is generally reported that pressure can alter processes of translation, inducing or eliminating production of particular proteins. [49] What might also have happened in performed experiment – the expression of β-galactosidase gene could have been altered or even silenced.

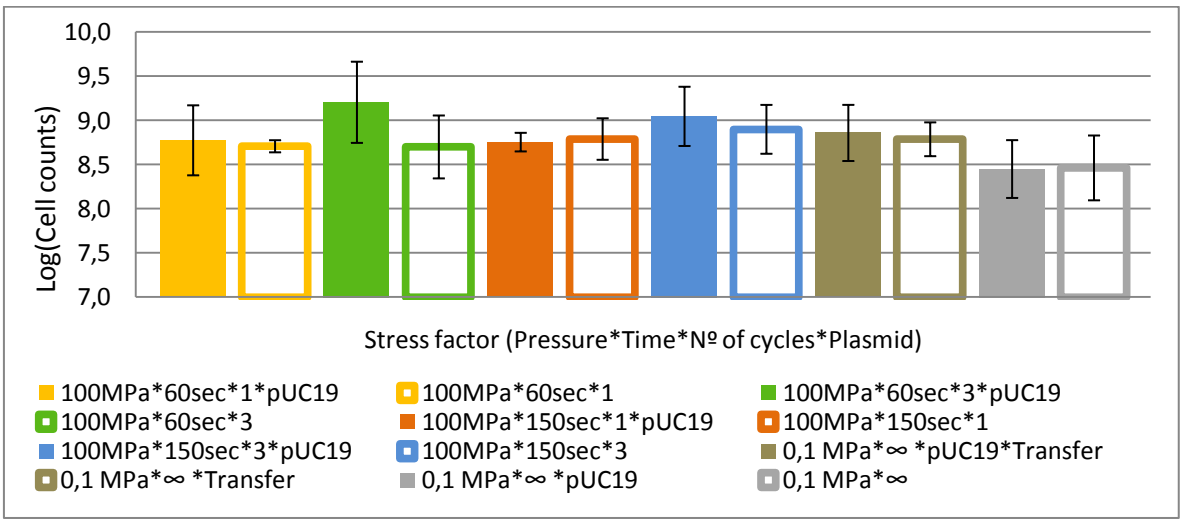
Consulting the work by Del Olmo [63], it was recognized that multiple-cycle pressurization could possible bring improvement to the innovative methodology.

3.2.5. Impact of single- and multiple-cycles

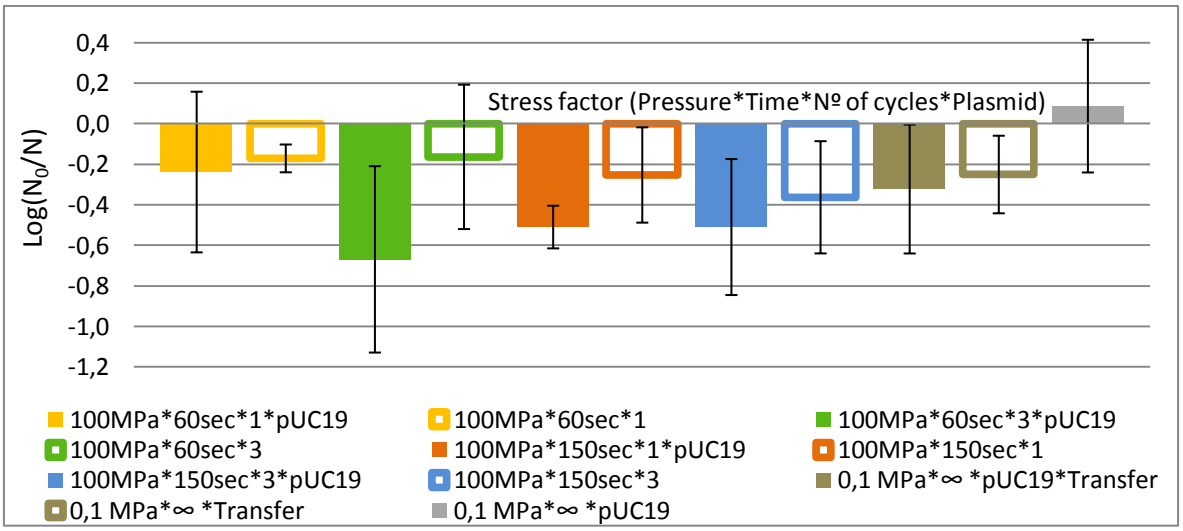
The aim of assay was to analyze the effect of pressurization in cycles. The experiment was carried at 100 ± 5 MPa since results indicated the value as potentially the most promising. Treatments were performed during 60 seconds and 150 seconds and each time was tested at single and multiple cycles. Cycles were defined as equal time intervals with 5-second pauses between. In the effect, schemes of pressurization time could be

outlined as (20`` - 20`` - 20``) and (50`` - 50`` - 50``) for respectively 60- and 150-second long treatments. As previously, the double samples were subjected to pressurization: with and without plasmid. In order to determine the influence of transfer step in handling procedure, additional blanks were prepared and placed in 400 µL Eppendorf tubes. The results are presented in the table 9 (Appendices, p. 64).

Graph 12 visualizes viability of cells in relation to the stress factor defined as combined influence of: pressure, time and number of cycles. On both graphs, bars filled with color correspond to samples where pUC19 was added and contoured reflect treatment to which exclusively cell suspension was subjected.



Graph 12 Results of cell viability after the treatment depending on stress factor (Pressure*Time*Nº of cycles*Plasmid).



Graph 13 Decimal reduction of cells caused by treatment depending on stress factor (Pressure*Time*Nº of cycles*Plasmid).

Analyzing graphs 12 and 13 could be noticed that longer treatment time resulted in decreased viability of cells. The observation was valid for both tested cycle variants. Interestingly, the impact of applied 3-cycle pressurization was found as a factor "softening" the applied stress giving increased numbers of survivors in comparison to the 1-cycle.

Similarly to previous results, the observed fluctuations associated with the presence of pUC19 were negligible. Hence, the plasmid was confirmed as having minor impact on the bacteria viability.

Negative values of decimal reduction found on the graph 13 implied cell growth in treated samples. However, the experiment included also analysis of unpressurized but transferred to 400 µL Eppendorf tube blank sample (0,1 MPa*∞*Transfer) where the increase of number of colony-forming units was observed as well. Hence, it proved that the contamination was probably introduced at this step of handling.

After 24-hour incubation at 37^{o}C there was no indication of cell growth on selective plates. Prolonged to 48 hours incubation time resulted in observation of morphologically heterogonous colonies. Petri dishes with observed microbial growth are presented on figures 9 - 13.

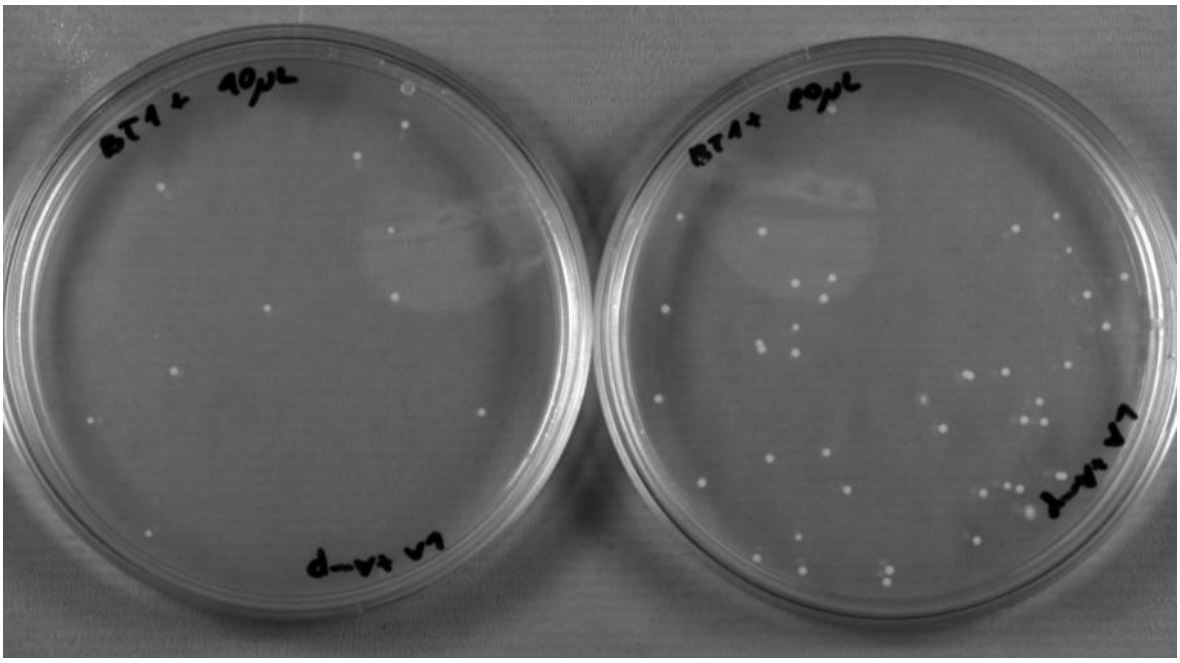


Figure 9 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Blank i.e. not pressurized *E. coli* and pUC19 sample transferred to 400 µL Eppendorf tube (0,1 MPa, ∞); Volume plated: 40 (left plate) and 80 µL (right plate).

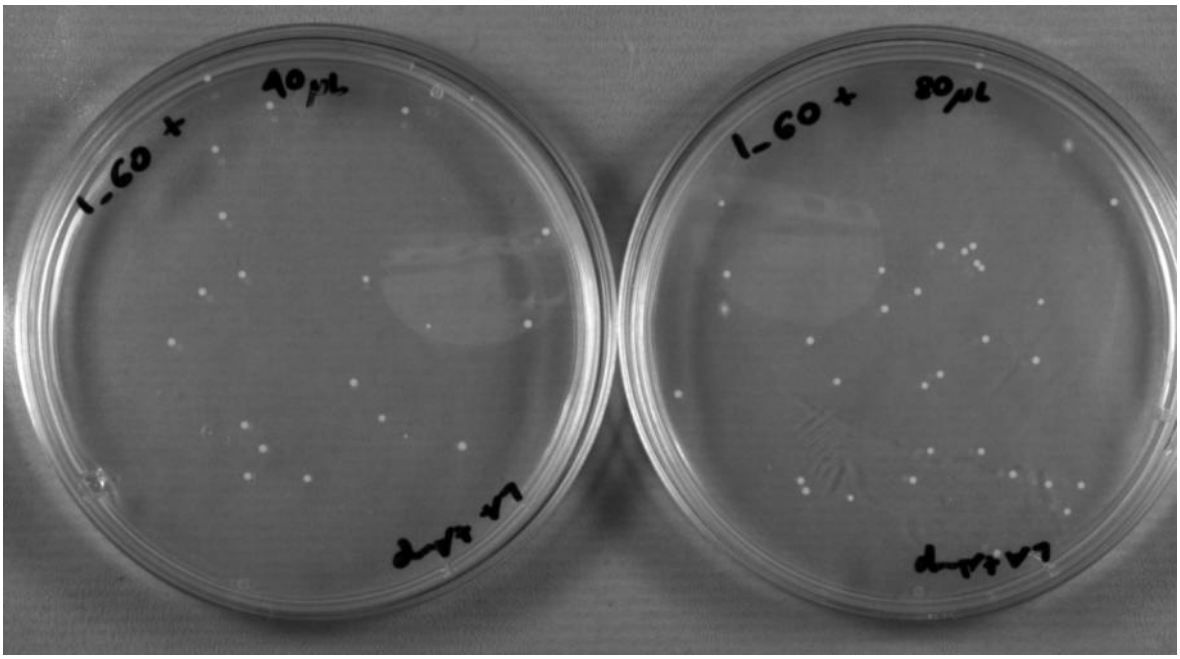


Figure 10 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 100±5 MPa, 60 sec., 1 cycle; Volume plated: 40 µL (left plate) and 80 µL (right plate).

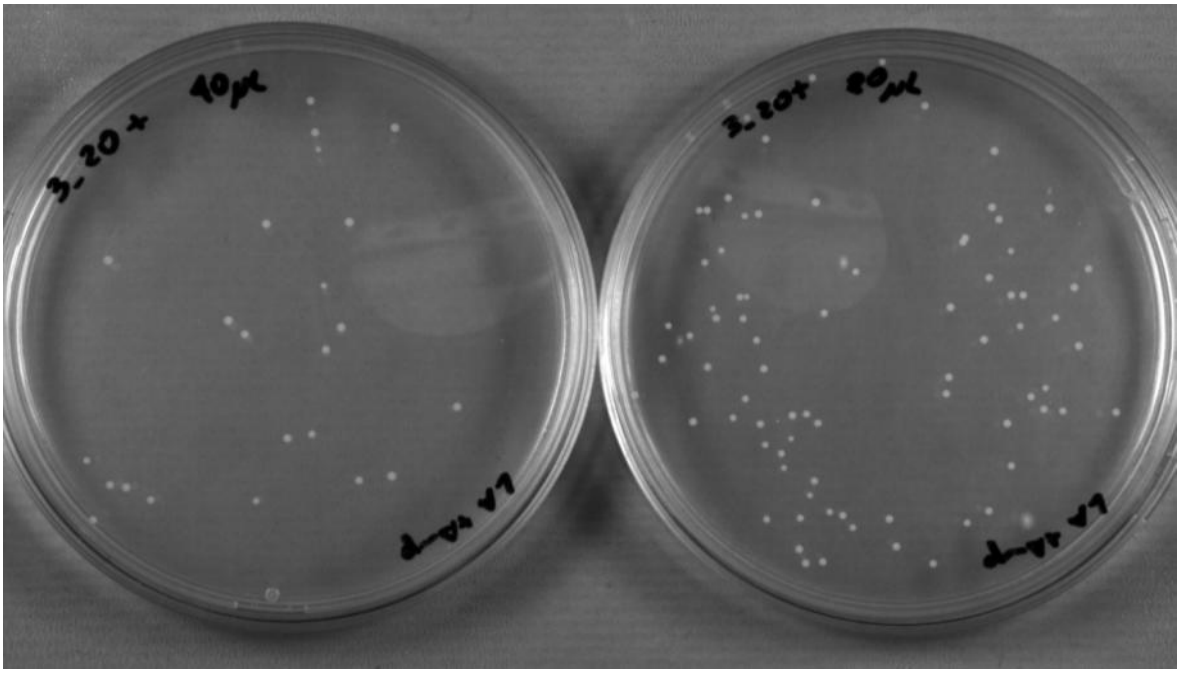


Figure 11 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 100±5 MPa, 60 sec., 3 cycles; Volume plated: 40 µL (left plate) and 80 µL (right plate).

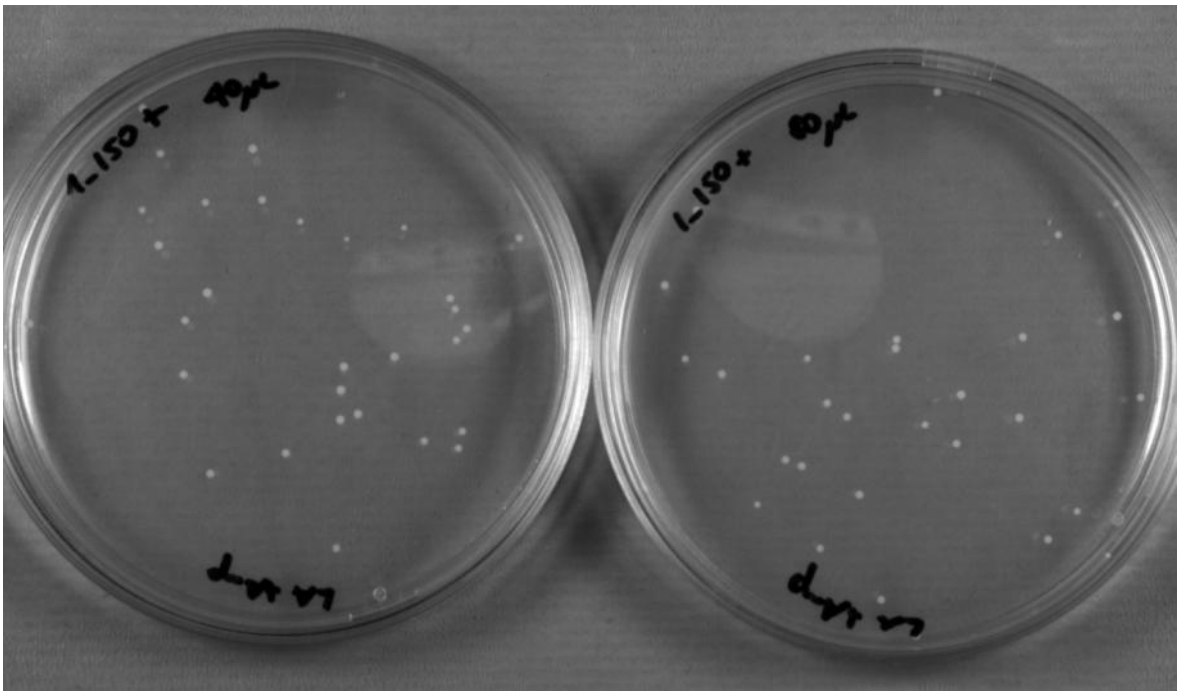


Figure 12 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 100±5 MPa, 150 sec., 1 cycle; Volume plated: 40 µL (left plate) and 80 µL (right plate).

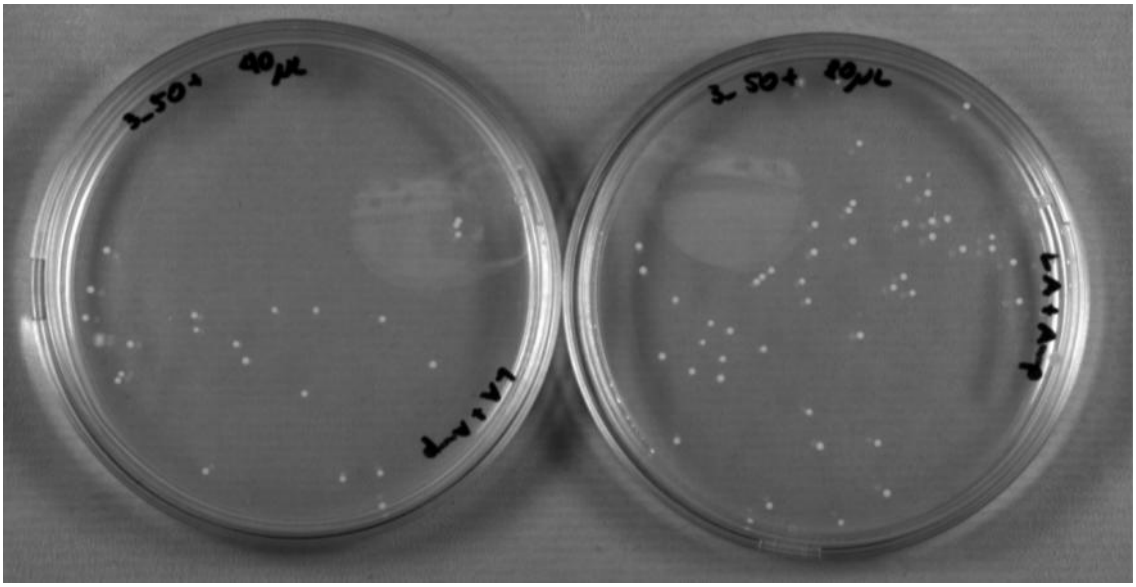


Figure 13 Selective Petri dish (LA + Amp) after 48 hours of incubation at 37ºC; Applied treatment: 100±5 MPa, 150 sec., 3 cycles; Volume plated: 40 µL (left plate) and 80 µL (right plate).

When incubation at 37^{o}C was prolonged to 48 hours, it was possible to observe cell growth on medium with antibiotic. In accordance to figures 9 - 13, the number of formed colonies was related to treatment and plated volume. However, the correlation was more

moderate than recorded previously because of lower density of growth and microorganisms could not be ambiguously recognized as *E. coli* TOP.

In order to identify formed on selective plates colonies, microscopic observation was performed. Majority of observed cells were morphologically similar to *E. coli* assuming shape of short rods. However, several individuals differed from the rest, indicating to shape-changing activity of pressure or presence of possible contaminants. The population was recognized as alive and well distributed. Absence of aggregates was in accordance with reported dispersing effect of high pressure on clusters. [49] Among cells subjected to different treatments, any visible and significant difference could be found.

Similarly to previous experiment, blue/white screening of microorganisms was performed but with equal result – exclusively growth of white colonies was observed. Furthermore, attempts to extract the plasmid with GeneJET Plasmid Miniprep Kit and Genomic DNA Purification Kit (Fermentas) were accomplished without success. Also after improvement of procedures with lysozyme digestion the cell breakage was not realized.

The identification of colonies grown on selective plates after 24-hour incubation was achieved with microscopic observation after Gram staining, bringing the experimental work to the end. Cultures subjected to the analysis included non-competent *E. coli* TOP10 as a reference, cells derived from the blank and cells treated with high pressure (treatment with 1 and 3 cycles).

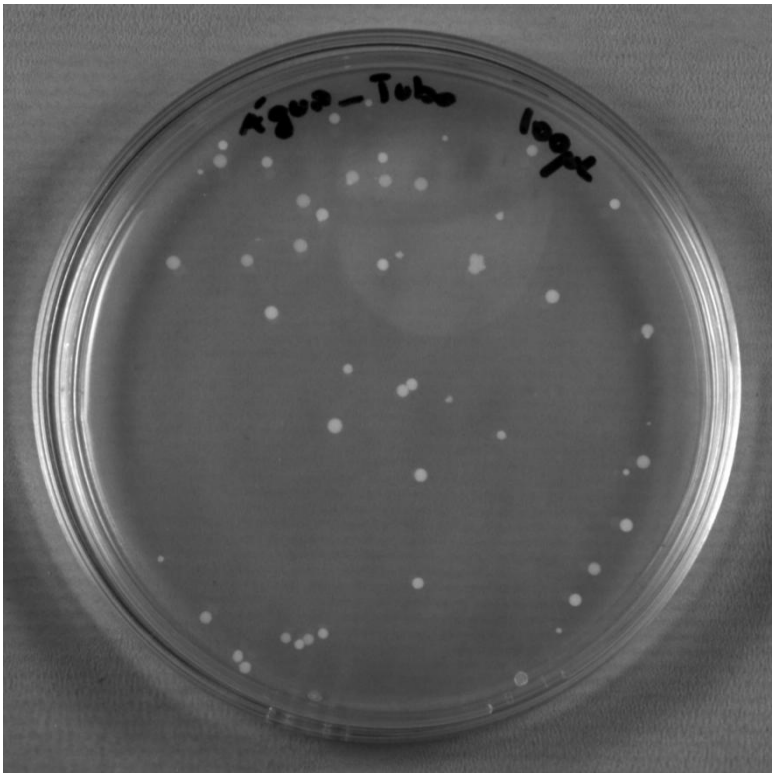
In the result, the initial strain appeared as purple small rods confirming that observed microorganisms are Gram-negative bacteria. On the contrary, all the rest specimens accepted navy-blue coloration specific for Gram-positive individuals. However, regard to the morphological characteristics they were identified as yeasts.

Re-observation of Petri dishes with selective medium after 2-week storage at $4^{o}C$ concurs with the conclusion. Colonies formed on the plates with pressurized samples appeared more transparent than those grown from initial culture of non-competent bacteria – clearly white. More, the smell characteristic for *E. coli* could not be detected on dishes prepared after treatment indicating absence of transformed host organism.

Identification of microorganisms as possible yeast contamination explains failure of lysis and growth on medium with antibiotic.

Increase in number of microorganisms after pressurization could be caused by disintegration of cellular aggregates or activation of growth. Interestingly, similar phenomena was observed in experimental work on *Pichia stipis* by Almeida [64]. According to the report, the yeast growth was noted under 50 MPa and 100 MPa during respectively 2,5 minutes and 7,5 minutes at 37,5^{o}C.

To determine probable origin of the contamination additional simulation of experiment was realized. In analysis 400 µL Eppendorf´s tubes were filled with distilled water and followed standard experimental procedure analogous as for blanks – without pressurization. Thus, specimens were pipetted to tubes, transported to the laboratory with High Pressure equipment forth and back, and plated on non-selective LA medium. After 48-hour incubation at 37ºC, formed colonies could be observed. The photo of Petri dish with obtained growth is shown on figure 14.



F**igure 14** Non-selective Petri dish (LA) after 48 hours of incubation at 37^{o}C; Samples of distilled water subjected to standard experimental procedure for blanks – not pressurized; Volume plated: 100 µL.

Observation of colony-forming units on selective medium indicates that 400 µL Eppendorf´s tubes are potentially source of contamination. Material applied in high pressure treatments, as non-autoclavable was sterilized with UV radiation. This method

may face unsatisfying efficiency since it has low penetrating power. Utilization of other technologies improving asepsis of tubes should be considered in the future. Solution could be also sterilization with application of several combined methods.

In conclusion, considering that the observed growth on selective plates was possibly representative of yeasts, presence of *E. coli* transformants was not proved. Use of another microorganism/plasmid could be tried in future experiments. Also, it must be kept in mind that treatments were carried out with cells with no previous preparation. In electroporation protocol for example, cells are made electrocompetent before. And more importantly, the decimal reductions imparted by the pressure treatments were small, indicating a low level of damage to cells in general, what might not be enough to create the necessary cell permeability, so that plasmid uptake can occur.

4. Conclusions and Proposals for future

Initially the transformation efficiency for selected host-plasmid pair was determined performing electroporation. The rate was defined for two values of plasmid concentration applied during experimental work: 0,01 µg/mL and 100 µg/mL. In the result obtained transformation efficiencies were in order of magnitude of transformants/µg of DNA for both concentrations of pUC19.

In the effect of adjustment of treatment conditions, pressure in the range 50 – 200 MPa was selected as the most adequate to work considering influence on viability of *E. coli* TOP10. Pressurizations should not exceed approximately 1 minute on single-cycle procedure and could be prolonged for multiple-cycles. Due to cell recovery, addition of SOC was suggested to be not-immediate. Treatments carried at normal compression rate were recognized as less severe for bacteria. All those conditions were established considering viability of cells.

It must be remembered that each assay was carried only once and so the number of replicates should be preferably increased to give more reliable values. However, obtained preliminary observations and results may be valuable indications for future works on genetic the transformation by high hydrostatic pressure.

Despite there exist several theoretical indications suggesting that high hydrostatic pressure could be successfully employed as transformation technology, performed experimental work did not provide any proof. It is essential to remember that transformation is a complex process and it depends on many factors. Thus, it could be suggested to test another variables and improvements. Progression may be brought by for example different host-plasmid pairs, particular conformations and sizes of introduced DNA or the type of solvents used. Also, the methods of preparation of microorganisms could be taken into consideration with the special attention to induced capabilities, type of resuspension liquid and phase of cell growth. In addition, higher pressure stressing treatments might be used, to impart more serious damage to cells to increase membrane permeability.

Considering all available opportunities to improve the here studied innovative methodology, I am full of optimism and I do believe that high hydrostatic pressure may bring highly effective solution and progress to genetic engineering.

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Table 1 Available technologies of transformation and their evaluation [8, 10-17]

Method	Principle of action	Typical efficiency rate [CFU/µg DNA]	Main pros and cons
Chemotransformation	Membrane permeabilization itself is caused by temperature shock but there are several possible chemicals enhancing the transformation (e.g. cations, ethanol or cyclodextrines) when added to DNA-cell mixture.	10^5-$2x10^9$	⊕ One of the most broadly applicable; ⊕ Accessible; ⊕ Inexpensive; - Hazard of chemicals - toxic in high concentrations; - Success depends on purity of chemicals.
Electroporation	Electric pulse generates pores in cell membrane permitting transport of particles through it.	$0,5$-$5x10^{10}$	⊕ One of the most broadly applicable; ⊕ Highly efficient; ⊕ Influenced by many factors thus may be successfully optimized controlling different parameters; ⊕ Quick and easy; - Requires individual optimization for particular cells; - Demands removal of unnecessary ions.
Freeze and thaw	Phase transition in membrane induces pores through which molecules can pass.	$\sim10^3$	⊕ Easy; - The least efficient.
Sonoporation	Membranes are physically permeabilized by the "cavitation bubbles" generated in a liquidous environment with ultrasound.	$\sim10^7$	⊕ Broadly applicable; ⊕ Less limited by ionic strength and voltage than electroporation; ⊕ Operates in room temperature, in any culture medium; ⊕ Easy to scale-up.

Liposome-mediated	DNA enclosed in liposomes is delivered to the host when phospholipid carrying structure attaches and unites with inner membrane.	~$2x10^8$	⊕ Rather efficient; - Requires prior removal or permeabilization of cell wall; - Application limited to animal and plant cells.
Chitosan-mediated	Chitosan interacts with DNA chains creating ionic complexes easily transported by endosomes through membranes.	No data found	- Toxicity of the chitosan for bacteria.
Biolistic	Bombardment of the cell with small particles carrying DNA.	$2x10^2$-$8x10^8$	⊕ Varied improved alternatives are available (tungsten, golden, magnetic nanoparticles, electrospray etc.), ⊕ Used for animal and plant cells; - Application in bacteria is impractical because of the cell dimensions.
Tribos	Based on the effect of Yoshida. Fibers carrying the DNA interrupt cell membrane employing friction forces and in the effect deliver external genes to the host.	10^4-10^6	⊕ Utilizes stationary phase, non-competent cells; ⊕ Sinchronic transformation and plating; ⊕ Rather quick and simple; ⊕ Inexpensive; - Recent and still under progress.

Table 5 Results of transformation efficiency for electroporation of electrocompetent *E. coli* TOP10 with average values and standard deviation (STDV).

Concentration of plasmid	Voltage	Time constant	Dilution	Volume plated	Cell counts	Amount of DNA	Efficiency	Average efficiency	STDV
[µg/mL]	[kV]	[msec]	-	[µL]	[cfu/plate]	[µg/plate]	[Transformants/µg of DNA]	[Transformants/µg of DNA]	[Transformants/µg of DNA]
0,01	1,80	5,1	-	40	79	7,26E-07	1,09E+08	9,47E+07	2,00E+07
				80	117	1,45E-06	8,06E+07		
	1,80	4,6	-	40	50	7,26E-07	6,89E+07	6,30E+07	8,28E+06
				80	83	1,45E-06	5,72E+07		
100	1,80	5,2	1:1000	40	192	1,33E-05	1,44E+07	1,18E+07	3,72E+06
				80	244	2,66E-05	9,18E+06		
	1,80	5,2	1:10000	40	51	1,33E-06	3,84E+07	3,44E+07	5,59E+06
				80	81	2,66E-06	3,05E+07		

Table 6 Results obtained after treatment of non-electrocompetent *E.coli* TOP10 with stress factor (Pressure*Time): cell counts, viability and decimal reduction.

Stress factor (Pressure*Time)	Compression time	Temperature change	Cell counts [cfu/mL]	Log(Cell counts)	Log(N_0/N)
50 MPa*2,5 min	10''	20°C→20,5°C	4,14E+05	5,62	2,19
100 MPa*2,5 min	35''	20°C→21°C	4,38E+04	4,64	3,17
200 MPa*1 min	40''	20°C→21,4°C	1,00E+04	4,00	3,81
300 MPa*1 min	50''	20°C→21,8°C	3,38E+04	4,53	3,28
400 MPa*1 min	1' 30''	20°C→22°C	1,48E+04	4,17	3,64
0,1 MPa*∞ (+pUC19)	-	≈0°C	6,74E+06	6,83	0,98
0,1 MPa*∞	-	≈0°C	6,42E+07	7,81	0,00

Table 7 Results obtained after treatment of non-electrocompetent *E.coli* TOP10 with stress factor (Pressure*Time*Compression rate): average values of cell counts and viability and their standard deviations (STDV).

				SOC supplemented after delay						**SOC supplemented immediately**		
				0 days of storage at 4°C			**2 days of storage at 4°C**			**2 days of storage at 4°C**		
Stress factor (Pressure* Time* Compression rate)	**Time of compres-sion delay**	**Compres -sion time**	**Tempera ture change**	Cell counts [cfu/mL]	Log(Cell counts)	STDV Log(Cell counts)	Cell counts [cfu/mL]	Log(Cell counts)	STDV Log(Cell counts)	Cell counts [cfu/mL]	Log(Cell counts)	STDV Log(Cell counts)
50MPa *60sec*NCR	5``	15``	21,0° →21,9°C	8,57E+07	7,92	0,14	1,75E+07	6,98	0,58	5,57E+05	5,74	0,12
100MPa *60sec*NCR	5``	21``	21,0°C →21,8°C	3,67E+08	8,45	0,48	8,06E+06	6,61	0,67	8,57E+04	4,81	0,49
200MPa *30sec*NCR	4``	34``	21,2°C →22,4°C	2,56E+08	8,32	0,39	6,88E+06	6,79	0,30	6,51E+05	5,65	0,45
50MPa *30sec*NCR	4``	14``	21,0°C →21,9°C	1,69E+08	8,19	0,27	1,69E+07	6,50	1,15	3,14E+06	6,32	0,47
100MPa *30 sec*NCR	4``	20``	21,2°C →22,4°C	9,73E+08	8,90	0,39	3,03E+07	7,30	0,54	2,14E+06	6,22	0,38
200MPa *10sec*NCR	4``	34``	21,0°C →22,7°C	3,05E+08	8,47	0,16	1,68E+07	7,05	0,46	1,51E+06	6,10	0,32
50MPa *30sec*FCR	2``	7``	21,2°C →22,0°C	3,14E+08	8,36	0,51	3,44E+06	6,47	0,32	1,17E+06	5,95	0,37
100MPa *30 sec*FCR	2``	11``	21,0°C →22,7°C	5,09E+07	7,67	0,26	5,25E+06	6,57	0,51	1,75E+06	6,00	0,56
200MPa *10sec*FCR	2``	20``	21,0°C →23,4°C	2,07E+07	7,22	0,43	7,62E+05	5,87	0,16	2,22E+06	6,33	0,13
0,1 MPa*∞	-	-	~0°C →~21°C	-	-	-	2,72E+06	6,22	0,53	-	-	-

Table 8 Results obtained after treatment of non-electrocompetent E.coli TOP10 with stress factor (Pressure*Time*Plasmid): average values of cell counts, viability and decimal reduction with standard deviation (STDV).

Stress factor (Pressure*Time*Plasmid)	Time of compression delay	Compression time	Temperature change	Cell counts [cfu/mL]	Log(Cell counts)	Log(N_0/N)	STDV
50MPa*30sec*pUC19	5``	15"	21,3°C→22,2°C	6,39E+09	9,49	-1,14	0,63
50MPa*30sec				1,59E+10	10,13	-1,77	0,37
100MPa*30sec*pUC19	6``	24``	21,3°C→22,3°C	3,09E+10	10,36	-2,00	0,50
100MPa*30sec				5,18E+09	9,43	-1,07	0,67
200MPa*10sec*pUC19	5``	34``	21,3°C→22,7°C	8,11E+09	9,63	-1,27	0,69
200MPa*10sec				5,91E+09	9,49	-1,13	0,67
0,1 MPa*∞ *pUC19	-	-	~0°C→~21°C	1,90E+08	8,26	0,10	0,19
0,1 MPa*∞				2,28E+08	8,33	0,02	0,21

Table 9 Results obtained after treatment of non-electrocompetent *E.coli* TOP10 with stress factor (Pressure*Time*Nº of cycles*Plasmid): average values of cell counts, viability and decimal reduction with stndard deviation (STDV).

Stress factor (Pressure*Time*Nº of cycles*Plasmid)	Time of compression delay	Compression time	Temperature change	Cell counts [cfu/mL]	Log(Cell counts)	Log(N_0/N)	STDV
100MPa*60sec*1*pUC19	6``	13"	22,7°C→23,0°C	7,44E+08	8,78	-0,24	0,40
100MPa*60sec*1				5,14E+08	8,71	-0,17	0,07
100MPa*60sec*3*pUC19	-	-	22,5°C→23,3°C	2,24E+09	9,21	-0,67	0,46
100MPa*60sec*3				6,27E+08	8,70	-0,16	0,36
100MPa*150sec*1*pUC19	6``	21``	22,3°C→23,2°C	3,85E+08	8,76	-0,22	0,11
100MPa*150sec*1				4,42E+08	8,79	-0,25	0,23
100MPa*150sec*3*pUC19	-	-	22,4°C→22,9°C	8,56E+08	9,05	-0,51	0,34
100MPa*150sec*3				5,85E+08	8,90	-0,36	0,28
0,1 MPa*∞ *pUC19*Transfer	-	-	~0°C→~21°C	8,23E+08	8,86	-0,32	0,32
0,1 MPa*∞ *Transfer				6,44E+08	8,79	-0,25	0,19
0,1 MPa*∞ *pUC19	-	-	~0°C→~21°C	3,23E+08	8,45	0,09	0,33
0,1 MPa*∞				3,44E+08	8,46	0,07	0,37