Lucrări ştiinţifice **Zootehnie şi Biotehnologii**, vol. **40(1), (2007**), Timişoara

STUDY REGARDING EFFICIENCY OF INDUCED GENETIC TRANSFORMATION IN *BACILLUS LICHENIFORMIS* WITH PLASMID DNA

STUDIU PRIVIND EFICIENŢA TRANSFORMĂRII GENETICE INDUSE LA *BACILLUS LICHENIFORMIS* CU ADN PLASMID

VINTILĂ T.
Faculty of Animal Science and Biotechnology, Timişoara, Romania

A strain of Bacillus licheniformis was subject to genetic transformation with plasmid vectors (pLC1 and pNC61), using electroporation technique, protoplast transformation and bivalent cations ($CaCl_2$) mediated transformation. In the case of transformation by ***electroporation*** *of Bacillus licheniformis B40, the highest number of transformed colonies (3) were obtained only after a 1,79 KV electric shock, for 2,2 milliseconds. Using this transformation technique we have obtained six kanamycin resistant transformants. The frequency of Bacillus licheniformis B40* ***protoplasts*** *transformation using pLC1 and pNC61 plasmid vectors is approximately 10% (TF = 10%). As a result of pLC1 plasmid integration in Bacillus licheniformis protoplasts, six kanamycin resistant transformants were obtained. The pNC61 plasmid, which confers trimethoprim resistance, does not integrate in receiver cells by protoplast transformation. The direct genetic transformation in the presence of* ***bivalent cations*** *($CaCl_2$), mediated by pLC1 and pNC61 plasmid vectors, produce a low transformation frequency. Using this technique, we have obtained three trimethoprim resistant colonies and four kanamycin resistant colonies. The chemical way of transformation is the only technique, which realizes the integration of pNC61 in B. licheniformis B40 cells.*
Key words*: genetic transformation, Bacillus licheniformis, electroporation, protoplast, pLC1, pNC61.*

Introduction

The main part of enzymes used in industry, medicine, agriculture, food or feed industry is produced by microorganisms, in industrial biotechnology. A large part of these enzymes are produced by genetically modified microorganisms (GMM). Although many governments (including E.U.) oblige labeling of products containing GMM, their use did not decrease, mainly due to their economical importance and lack of alternatives (for example insulin, chimosin, amylases, proteases etc). The methods used in genetic engineering of industrial microorganisms include genetic

transformation. *Bacillus licheniformis* is a GRAS (generally recognized as safe) microorganism used in industry for protease production. Plasmids pLC1 and pNC61 are known for improvement of extra-cellular proteolytic activity of the host (Jurcoane 2000). During this experiment we will evaluate three transformation techniques (direct transformation, electroporation and transformation of protoplast) and their ability to insert above mentioned plasmids in *Bacillus licheniformis* cells for improvement of proteolytic activity of this strain.

Materials and Methods

Microorganism: one bacterial strain from the Collection of Industrial Microorganisms from Faculty of Animal Science and Biotechnology Timisoara, Romania was used in this study: *Bacillus licheniformisB40* a microorganism with proteolytic activity.

Methods:

1. Extraction of plasmid DNA from host-cells using ***Boiling miniprep method*** (Miller 1992).

Plasmids pLC1 and pNC61 were extracted from a strain of *Bacillus subtilis* in logarithmic growing phase in LB broth. The lysis buffer contains Tris, EDTA, Sucrose, Lysozyme, and BSA. The precipitation of cellular components and plasmid DNA was made using chloroform-isoamyl alcohol mixture and ethanol 95%. Elution buffer contains Tris and EDTA.

2. Digestion of plasmid DNA with restriction enzymes (Seidman 2000). Restriction was made using ECOR I enzyme.

3. Visualization of plasmid DNA molecules through agarose gel electrophoresys (Seidman 2000). Digested and undigested plasmid DNA was migrated in 1% agarose gel at 75 V and 60 mA. As marker was used λDNA digested with Pst I restriction enzyme.

4. Genetic transformation through electroporation (Phillip 1994). Three steps were followed in this phase: a) extraction of plasmid DNA from carrier cells; b) inducing competence in receiving cells using LB, LBSP and SHMG and testing of receiving cells for sensitivity to antibiotics used as markers in plasmids: kanamycin for pLC1 and trimethoprim for pNC61; and c) electroporation and genetic transformation of the competent cells with plasmid DNA using MicroPulser electroporator from BIO-RAD, 0.1 cuvette, electro-shock set at 1.8kV, 2.5 milliseconds. 100 µL of cell suspension after electroporation was inoculated in LB plates with kanamycin and trimethoprim and incubated at 37^{o}C 24 hours, until colonies appears.

5. Genetic transformation through protoplasts transfection (Phillip 1994). Three steps were followed in this phase: a) extraction of plasmid DNA from carrier cells; b) obtaining protoplasts of receiving cells using Penessay buffer, SMM buffer,

Lysozyme, DM3 agar and TES buffer; and c) transformation of protoplasts with plasmid DNA using SMM buffer, and PEG6000. 100 µL of cell suspension was inoculated in DM3 plates with kanamycin and trimethoprim and incubated at 37^{o}C 24 hours, until colonies appears.
6. Bivalent cations mediated (direct) genetic transformation (Phillip 1994). Three steps were followed in this phase: a) extraction of plasmid DNA from carrier cells; b) inducing competence in receiving cells using SP II medium and testing of receiving cells for sensitivity to antibiotics used as markers in plasmids: kanamycin for pLC1 and trimethoprim for pNC61; and c) genetic transformation of the competent cells with plasmid DNA using SMS, glucose, yeast extract, casamino acids and $CaCl_2$. 100 µL of cell suspension was inoculated in LB plates with kanamycin and trimethoprim and incubated at 37^{o}C 24 hours, until colonies appears.
7. Casein hydrolysis test (Zarnea, 1992). Casein agar plates were inoculated with transformed microorganism and after incubation the presence and size of the clear halo as a result of casein hydrolysis was evaluated and measured.

Results and Discussions

1. Regarding extraction and visualization of plasmid DNA in gel electrophoresis, figure 1 represents pLC1 plasmid undigested - fragments 1, 2, and 4, digested with PstI – fragments 5, and λDNA marker – fragments 3, from 2000 to 9400 bp.

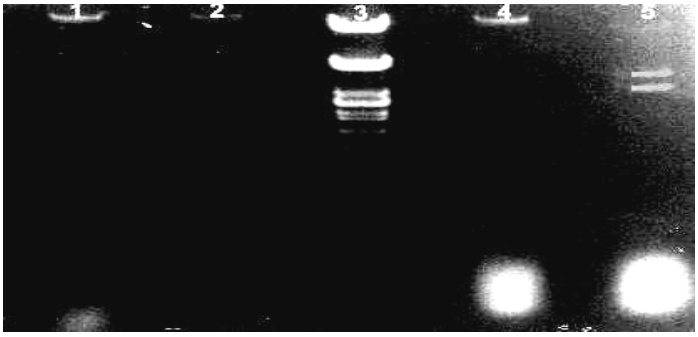

Figure 1. Visualization of pLC1 plasmid DNA in agarose gel

2. Regarding transformation by electroporation of the strain *Bacillus licheniformis B40*, four probes were electroporated: A – 1,8 kV; 2,5 milliseconds; B – 1,8 kV; 2,2 milliseconds; C – 1,79 kV; 2,2 milliseconds; D – 1,8 kV; 2,5 milliseconds. The highest number of transformed colonies (3) were obtained only after a 1,79 KV electric shock, for 2,2 milliseconds. Using this transformation technique we have obtained 6 kanamycin resistant transformed colonies.

3. Regarding transformation of protoplasts, during protoplasization the number of viable cells decreased very much, from a range of 10^6 cells / mL to 80 viable cells / ml after treating with SMM buffer and Lysozyme. Figure 2 presents *B. licheniformis* cells and protoplasts.

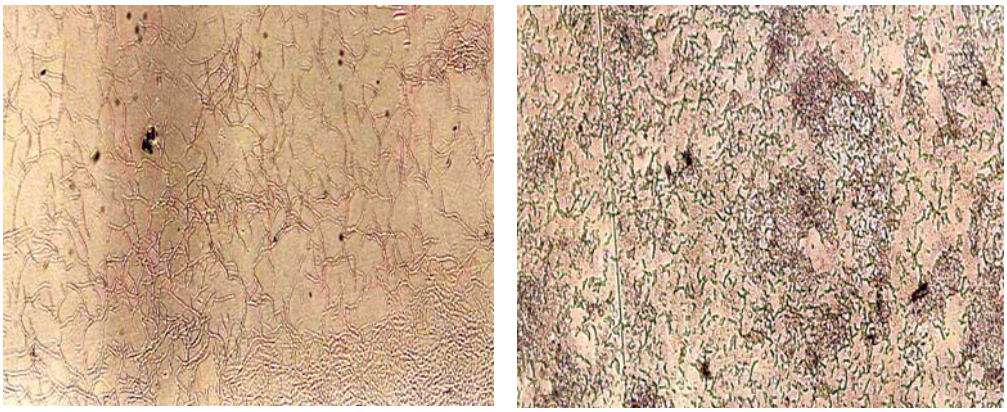

Figure 2. Microscope image of *B. licheniformis* cells (left – cells before, and right – cells after protoplastization).

The frequency of *Bacillus licheniformis B40* protoplasts transformation using pLC1 and pNC61 plasmid vectors is approximately 10% (**TF = 10%**). As a result of pLC1 plasmid integration in *Bacillus licheniformis* protoplasts, 6 kanamycin resistant transformants were obtained. The pNC61 plasmid, which confers trimethoprim resistance, does not integrate in receiver cells by protoplast transformation.

4. The direct genetic transformation in the presence of bivalent cations ($CaCl_2$), mediated by pLC1 and pNC61 plasmid vectors produce a low transformation frequency. Using this technique, we have obtained three tromethoprim resistant colonies and four kanamycin resistant colonies. The direct transformation is the only technique, which realizes the integration of pNC61 in *B. licheniformis B40* cells.

5. Testing the obtained genetically transformed microorganisms. The qualitative method for determination of proteolytic activity consists in dispersion of bacterial cells on the surface of agar plates containing casein. After 24 hours of incubation at 37ºC the clear halo (hydrolysis of casein) around the bacterial single colonies is measured (fig. 3).

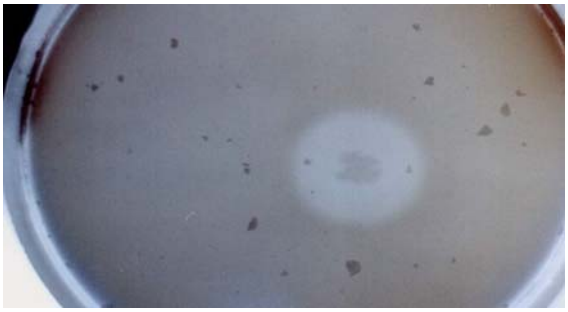

Figure 3. Halo produced by a *B. licheniformis* transformed colony, kanamycin-resistant on casein-agar medium

High secretions of extra cellular proteases produce a large clear halo. The proteolytic activity of the 18 transformants obtained using the three transformation techniques, is lower, equal, or a slightly higher then the proteolytic activity of *Bacillus licheniformis B40* parental strain. In 13 transformants, a positive proteolytic reaction was evident using the casein hydrolysis test. Four transformants present proteolytic activities lower, or approximately equal with parental strain activity. The *B. licheniformis Tp2* (protoplast transformed) reveals a visible improvement of proteolytic activity.

Conclusions

1. In case of transformation by electroporation of the strain *Bacillus licheniformis B40*, the highest number of transformed colonies (3) were obtained only after a 1,79 KV electric shock, for 2,2 milliseconds. Using this transformation technique we have obtained six kanamycin resistant transformants;
2. The frequency of *Bacillus licheniformis B40* protoplasts transformation using pLC1 and pNC61 plasmid vectors is approximately 10% (**TF = 10%**). As a result of pLC1 plasmid integration in *Bacillus licheniformis* protoplasts, six kanamycin resistant transformants were obtained. The pNC61 plasmid, which confers trimethoprim resistance, does not integrate in receiver cells by protoplast transformation.
3. Direct genetic transformation in the presence of bivalent cations ($CaCl_2$), mediated by pLC1 and pNC61 plasmid vectors, produce a low transformation frequency. Using this technique, we have obtained three trimethoprim resistant colonies and four kanamycin resistant colonies. The chemical way of transformation is the only technique, which realizes the integration of pNC61 in *B. licheniformis B40* cells.

Bibliography

1. **JURCOANE S.,** *Biotehnologii. Fundamente, bioreactoare, enzyme.* Editura Tehnica Bucuresti 2000;
2. **MILLER J. H.,** *A short course in bacterial genetics. A laboratory manual and handbook for Escherichia coli and related bacteria.* Cold Spring Harbor Laboratory Press, 1992;
3. **PHILLIP G., MURRAY R.G.E., WOOD W.A., KRIEG N.,** *Methods for general and molecular bacteriology.* American Society for Microbiology, Whashington 1994.
4. **SEIDMAN L. A., MOORE C.J.,** *Basic laboratory methods for biotechnology. Textbook and laboratory reference.* Prentice-Hall, 2000.
5. **ZARNEA G., MIHAESCU GR., VELEHORSCHI V.,** *Principii si tehnici de microbiologie generala*. Facultatea de Biologie Bucuresti 1992.

STUDIU PRIVIND EFICIENŢA TRANSFORMĂRII GENETICE INDUSE LA *BACILLUS LICHENIFORMIS* CU ADN PLASMID

VINTILĂ T.
Facultatea de Zootehinie si Biotehnologii, Timişoara, Romania

O tulpină de Bacillus licheniformis a fost supusă transformării genetice cu ADN plasmidial (pLC1 şi pNC61), folosind tehnicile de electroporare, transformare de protoplaşti şi transformare directă în prezenţă de cationi bivalenţi ($CaCl_2$). În cazul transformării prin electroporare a tulpinii Bacillus licheniformis B40, cel mai mare număr de colonii transformate (3) a fost obţinut aplicând un şoc electric de 1,79 KV timp de 2,2 milisecunde. Cu ajutorul acestei tehnici noi am obţinut 6 transformanţi rezistenţi la canamicină. Frecvenţa transformării protoplaştilor de Bacillus licheniformis B40 cu vectorii plasmidiali pLC1 şi pNC61 este în jur de 10% (TF = 10%). Ca urmare a tehnicii de transformare a protoplaştilor, au fort obţinuţi 6 transformanţi de Bacillus licheniformis rezistenţi la canamicină. Plasmida pNC61, care are ca marker rezistenţa la trimetoprim, nu s-a integrat în celulele receptoare prin transformarea protoplaştilor. Transformarea genetică directă în prezenţă de cationi ($CaCl_2$), mediată de vectorii plasmidiali pLC1 şi pNC61 se produce cu o frecvenţă foarte scăzută. Cu ajutorul acestei tehnici am obţinut 4 transformanţi rezistenţi la canamicină şi 3 transformanţi rezistenţi la trimetoprim. Este însă, singura metodă cu ajutorul căreia s-a putut insera plasmida pNC61 în celulele de B. licheniformis B40.

Cuvinte cheie: transformare genetică, Bacillus licheniformis, electroporare, protoplast, pLC1, pNC61.