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ASSESMENT OF CRYOPRESERVATION SYSTEMS INFLUENCE ON THE SURVAVIAL OF *E. COLI* RECOMBINANT STRAINS

TESTAREA INFLUENȚEI SISTEMELOR DE CRIOCONSERVARE ASUPRA VIABILITĂȚII TULPINILOR RECOMBINANTE DE TIP *E. COLI*

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The cryopreservation systems of recombinant bacterial cells based on glycerol were studied in these experiments according to the hypothesis that glycerol is one of the widely used cryoprotective additives in microbiology and a multitude of factors affecting the effectiveness of cryopreservation in microorganisms; the best cryoprotective additive and the optimum concentration for a particular microorganism has to be determined empirically. The results obtained in this experiment are showing that the freezing procedure at -80°C in LB 40% glycerol is the optimum system for the cryopreservation of E. coli DH5α recombinant cells. The use of SOC medium supplemented with 10g/l NaCl provided more proper conditions of culture for the defrosted E. coli DH5α recombinant cells, reducing the osmotic stress during the recovery after thawing. The utilization of this optimum cryopreservation system offer the possibility of preserving the large volume of work and time involved by the recombinant DNA technology procedures applied for obtaining a recombinant strain, avoiding the storage of recombinant strains by costly and time consuming microbiology culturing techniques.

Key words: cryopreservation system, recombinant bacteria survival, bacteria culture.

Introduction

The discovery of new cryopreservation reagents that protect eukaryotic cells against freezing damage marked the beginning of modern cryotechnology. The cryoprotective additives used in the frozen storage of microorganisms include a variety of simple and more complex chemical compounds, but only a few of them have been used widely and with satisfactory results. The best cryoprotective additive and the optimum concentration for a particular cryosensitive

microorganism has to be determined empirically. There are a multitude of factors that affect the effectiveness of cryopreservation in microorganisms such as the specie, strain, cell size and form, growth phase and rate, incubation temperature, growth medium composition, pH, osmolarity and aeration, cell water content, lipid content and composition of the cells, density at freezing, composition of the freezing medium, cooling rate, storage temperature and duration of storage, warming rate, and recovery medium.

The most generally and widely used cryoprotective additives in microbiology are Me2SO and glycerol. While polyhydric alcohols, especially glycerol, are commonly used as cryoprotective additives, the use of monovalent alcohols is comparatively infrequent, probably due to their toxicity for many biological systems as is well known. The cryoprotective effect of glycerol was discovered much earlier than is usually stated: Keith observed that an addition of 5–42% glycerol to suspensions of E. coli in water permitted long-term survival of this bacterium at -20°C.

Glucose has been used in cryomicrobiology at concentrations 1–18% (median 4%). Improved survival of certain bacterial cultures at -20°C using glucose solutions was described very early.

Some scientist came to the conclusion that the cryoprotective effect of some cryoprotective additives on cellular survival and viability does not depend significantly on cooling rate, at least in the considered range. However, it is always very important to take the toxicity of individual cryoprotective additives for particular microorganisms into consideration. It has been suggested that bacterial strains behavior could explain the strong deviations from the ideality observed in cryopreservation systems, and could be related to their biological properties, particularly to the cryoprotective ability.

Materials and Methods

Biological material

The *E. coli DH5α* recombinant cells (containing the ovine leptin gene coding sequence inserted in the pGEMT easy vector) was used as biological material in the cryopreservation laboratory experiments of the recombinant strain. The ovine leptin gene isolated (from adipose subcutaneous tissue) by a specific RT-PCR reaction (with specific primers encoding the restriction site of the enzymes EcoRI and BamHI) and a further purification process by cutting its band from the 1% agarose gel, was used in the CaCl2 bacterial transformation of *E. coli DH5α* host cells.

E. coli DH5α recombinant bacteria culture

Bacteria were inoculated in a liquid culture at 37 °C, for 24 hours, aerobically in tubes containing 5 ml LB growth medium; the LB growth medium contained: 10 g tryptone, 10 g yeast extract and 5 g NaCl per liter (Sigma).

The liquid culture was used to streak the bacteria cells on LB agar 50 mM ampicillin plates, that were subsequently incubated at 37°C, for 24 hours. The colonies grown on The solid medium supplemented with ampicillin antibiotic sustain only the growth of the recombinat *E. coli DH5α* cells (which has the amplicillin resistance gene of the pGEMT easy vector harboring the coding sequence of the ovine leptin gene). Solid medium for bacteria plates were prepared by the same ingredients used to make the liquid medium and 15 g of agar per liter and ampicillin (in a final concentration of 50 mM) that were added; than the solid medium was poured into 90 mm Petri dishes.
A single colony was transferred to a tube containing 5 ml LB growth medium using sterile technique and incubated 37°C, for 24 hours. Plasmidial DNA was extracted from the liquid culture using the Wizzard Plus SV Minipreps DNA Purification System purchased form Promega according to the spin protocol of the manufacturer. To control the presence of the insert in the pGEMT easy vector, the plasmidial DNA extracted was migrated in 1% agarose gel electrophoresis.

Cryopreservation of *E. coli DH5α* recombinant strain

500 µl from the recombinant *E. coli DH5α* liquid culture were mixed to an equal volume of each variant cryopreservation medium. A total amount of 1 ml aliquots are transferred to plastic tubes for freezing; to avoid ice crystallization or to reduce crystal sizes, a very fast cooling was performed for each sample at -20°C and -80°C. The cryopreservation medium contained 10%, 20%, 30%, 40%, 50% and 60% glycerol in LB medium. Glycerol (1,2,3-propanetriol), is one of the most widely used cryoprotective additive in microbiology; it decrease the freezing-point of water and biological fluids by colligative action.
The bacterial cells were stored at the above temperatures for one week; forward an evaluation of the survival bacterial cells was performed.

Evaluation of the *E. coli DH5α* recombinant bacterial cells survival

100 µl of the defrosted bacteria suspension were inoculated in 5 ml SOC medium supplemented with NaCl and incubated at 37 °C, for 24 hours. The SOC medium contained: 2 g tryptone, 0,5 g yeast extract, 0,05 g NaCl, 10 µl 1M $MgCl_2$, 10 µl 1M $MgSO_4$ and 20 µl 20% glucose per 100 ml. As many cells, especially eukaryotic ones, are quite sensitive to osmotic shock, the frozen bacterial cells were suspended in SOC medium supplemented with 10 g/l NaCl.
Survival and viability were determined immediately after thawing by counting colony forming units (cfu), 10µl from the above liquid culture being plated on a LB agar 50mM ampicillin medium. For comparing the results five-tubes for each sample were used.

Results and Discussions

The cryopreservation systems of has been studied to optimize this process regarding the concentration of the cryoprotective reagent (glycerol) directed to a freezing (-20°C) or an ultrafreezing (-80°C) system according to the survival rate of the cells.

The 3456 bp band resulted in the 1% gel electrophoresis correspond to the size of the DNA fragment of the pGEMT easy vector harboring the coding sequence of the ovine leptin gene, extracted from the *E. coli DH5α* transformed colonies.

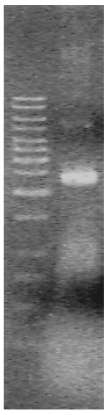

Figure 1. The transformed pGEMT easy vector (3456 bp)- migrated in agarose gel 1%: lane 1- 1 kb DNA Ladder (Fermentas), lane 2- the transformed pGEMT easy vector extracted from the recombinant E. coli DH5α

The survival of *E. coli DH5α* recombinant cells are summarized in the table 1.

Table 1

The C.F.U. number average grown the tested cryopreservation media in freezing and ultrafreezing systems

Cryopreservation medium composition and freezing condition	The average no. grown on LB agar 50mM ampicillin medium
LB 10% glycerol, -20°C	54
LB 20% glycerol, -20°C	62
LB 30% glycerol, -20°C	72
LB 40% glycerol, -20°C	104
LB 50% glycerol, -20°C	102
LB 60% glycerol, -20°C	94
LB 10% glycerol, -80°C	68
LB 20% glycerol, -80°C	82
LB 30% glycerol, -80°C	96
LB 40% glycerol, -80°C	120
LB 50% glycerol, -80°C	112
LB 60% glycerol, -80°C	98

The SOC medium supplemented with 10g/l NaCl utilization provide more proper conditions for the defrosted *E. coli DH5α* recombinant cells, reducing the osmotic stress during the recovery after thawing.

Conclusions

This paper describes an efficient system that was established for the storage in recombinant bacterial stocks in laboratory.

The cryopreservation systems of recombinant bacterial cells has been studied, and the results showed differences in the frozen storage of recombinant E. coli DH5α at -20°C or -80°C and regarding the glycerol content of the cryopreservation medium.

The results obtained in this experiment are showing that the freezing procedure at -80°C in LB 40% glycerol is the optimum system for the cryopreservation of *E. coli DH5α* recombinant cells.

In this paper are described suitable conditions for rapid rehabilitation of recombinant *E. coli DH5α* bacterial cells after thawing, the optimum cryopreservation system offering the possibility of preserving the large volume of work and time involved by the recombinant DNA technology procedures applied for obtaining a recombinant strain, avoiding the storage of recombinant strains by costly and time consuming microbiology culturing techniques.

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