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Genetic Transformation of Immobilized Competent Cells

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Abstract

This study describes the investigation of the possibility of genetic transformation of already immobilized competent cells by plasmids. The preliminary prepared competent cells were entrapped into granules of an insoluble carrier, a cryogel of poly(vinyl alcohol). The specific activity of organophosphatehydrolase and ampicillin resistance conferred by pOPf1 plasmid were used as markers of successful transformation of the immobilized competent cells. The effect of main experimental conditions of transformation usually used for free cells, i.e., time of incubation of cells with DNA solution, temperature, and time of heat shock, on the transformation efficiency of the immobilized competent cells has been studied. A number of important factors of preparation of immobilized transformed cells, i.e., the concentration of immobilized competent cells inside the granules, the concentration of DNA solution used for transformation, have been shown to affect the OPH-activity of the final immobilized transformants. The possibility of transformation of the immobilized competent cells by both single- and double-stranded plasmid DNA molecules has been demonstrated.

Index Entries: Immobilized cells; cryogel of poly(vinyl alcohol); genetic transformation; organophosphatehydrolase.

Introduction

Immobilized microbial cells used in biotechnology often represent genetically engineered strains. The fact that immobilization improves plasmid stability in genetically engineered microbial cells is well known now *(1,2)*. It was demonstrated by two scientific groups that immobilized cells are able to change some of their properties and, moreover, to acquire new

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features as a result of genetic transfer between cells *(3,4)*. There is nothing strange with it because the process of gene transfer is quite widespread in nature. Nothing is known so far about the possibility of transformation of immobilized cells.

It was shown that some bacterial strains of *Pseudomonas* and *Streptoccocus* cells immobilized within polysaccharide gel beads such as k-carrageenan and calcium alginate gel beads actively participate in gene-transfer-process like conjugation *(3,4)*.

The process of conjugation under such conditions is both the object for interesting scientific investigation and a good model to understand different aspects of microenviromental effects on the process of exchanging of genetic information between cells. These conditions almost imitate the same process in natural habitats when cells are entrapped into matrices or adsorbed on the surface of some natural supports.

Cell transformation is a much more useful and applied procedure for the production of new strains for industrial and scientific purposes than conjugation. The basic steps used by different biotechnologists to create new biocatalysts based on immobilized genetic engineered cells are as follows:

1. Preparation of competent cells and genetic materials for transformation;
2. Transformation;
3. Cultivation of free transformed cells in the selective medium;
4. Immobilization of gene-engineered cells; and
5. Application of the immobilized biocatalyst obtained for bioreactor processes.

The cell possesses an ability to accept exogenous genetic information only if it has increased cell wall permeability (competent cells). For this purpose, the cells collected at the definite growth phase are usually treated with buffers of certain pH and combination of ions.

In the case of immobilized cells, the transformation method developed in this work includes the following four stages:

1. Preparation of competent cells;
2. Immobilization of the competent cells obtained at the first step;
3. Transformation of the immobilized competent cells; and
4. Cultivation of the immobilized transformants.

As a rule, the competent cells are usually prepared in large amounts, frozen in liquid nitrogen, and stored in a refrigerator at –70°C until they are used for transformation *(5)*. Although the competent cells can be stored under these conditions for a fairly long time, the efficiency of their transformation decreases with the time of storage since the quantity of living cells among them is reduced.

We decided to make an attempt to immobilize competent cells inside the cryogel of poly(vinyl alcohol) (PVA). In this case, the cells to be stored also have to pass through the freezing stage, but this process will take place in the presence of PVA-cryogel, which is known to be a good

cryoprotector and could help the competent cells preserve their viability *(6)*. In principle, the presence of gel matrix could influence the ability of competent cells to be transformed by DNA molecules. However, it was shown previously *(7–9)* that the high-porous structure of PVA-cryogel allows the effective immobilized biocatalysts without considerable diffusion limitations to be created on the basis of various living microbial cells.

The proposed immobilization procedure could allow us:

1. To organize the storage of competent cells under more comfortable conditions,
2. To extend the total period of cell storage, and
3. To essentially increase the temperature of cell storage up to –20°C, the real temperature of cryogel formation.

The plasmid pOPf1 of special structure was created for transformation of the immobilized cells in this study. This plasmid, constructed on the basis of $pOP540\Delta_{2-29}$ *(10)*, contained the gene coding for the enzyme organophosphatehydrolase (OPH), hydrolyzing the phosphoorganic triesters with high specificity.

This enzyme was chosen because of its extremely high catalytic activity in paraoxon hydrolysis ($k_{cat} = 10^4$/s) *(11,12)* and the fact that *p*-nitrophenol accumulated as a product of this reaction is easily detected spectrophotometrically ($\lambda = 405$ nm).

In our previous studies *(9)*, it was shown that the OPH-activity of PVA-cryogel granules was directly proportional to the quantity of entrapped cells carrying the OPH expression plasmid. So the enzymatic activity was used as a parameter estimating the transformation efficiency of the immobilized cells.

The same plasmid also contained the gene for ampicillin resistance. Thus, there were two different markers to estimate the transformation efficiency of the immobilized competent cells.

Materials and Methods

Preparation of Competent Cells

The *Escherichia coli* strain C600 cells were cultivated in the SOB culture medium *(5)* until the beginning of the exponential phase of the cell growth, then centrifuged at 4000 rpm at 4°C for 10 min (JA-20 rotor, Beckman, Fullerton, CA) and then suspended in TFB buffer *(5)* followed by incubation on ice for 30 min. After the incubation, the cells were centrifuged again as described above, suspended in TFB buffer, followed by the addition of dimethylsulfoxide (DMSO) to the final concentration of 7%.

Immobilization of Competent Cells

The competent cells were centrifuged again and finally resuspended in 10% (w/v) aqueous PVA solution. The mass content of cells in the sus-

pension was 10% with the exception of the experiments with different ratios of PVA solution to cells. One hundred microliter aliquots of the cell suspension were placed into wells of a 96-well immunological plate. The plate was frozen and kept at –20°C for 16–20 h and then slowly thawed at room temperature. As a result of this treatment, the granules with the immobilized competent cells were formed at the bottom of each well.

Transformation of Immobilized Competent Cells

Double- and single-stranded forms of pOPf1 plasmid were used as a source of DNA material. Fifty microliters of plasmid DNA solution of various concentrations were added to each granule obtained, and the plate was incubated on ice for 1 h, followed by 42°C heat-shock treatment for 90 s in a water bath. After heat shock, the plate was placed on ice for 5 min and 1 mL of liquid SOB medium *(5)* was added to each portion of granules for cell adaptation after transformation.

Determination of Transformation Efficiency

The plasmids used for cell transformation contained the ampicillin-resistance gene as well as the gene coding for OPH. The acquisition of antibiotic resistance served as a marker of transformation process and was determined as follows. The granules with immobilized cells after cell adaptation for 1 h in the fresh medium without antibiotic were placed in the flasks with 200 mL of ampicillin containing SOB medium (70 μg/mL). The immobilized transformants were cultivated for 1–6 h at 37°C with agitation, and the free cell density was measured in the cultural medium at λ = 550 nm.

OPH enzymatic activity of free and immobilized cells was determined measuring the accumulation of *p*-nitrophenol in the reaction of paraoxon hydrolysis catalyzed by OPH-containing cells. One hundred microliters of 100 m*M* CHES buffer containing paraoxon (1 m*M*) were poured into the granules of immobilized cells after their preincubation at 37°C and the removal of SOB medium. The kinetics of *p*-nitrophenol formation was monitored, and OPH activity was measured. One unit of OPH activity is referred to as the activity of the amount of cells that is necessary for the formation of 1 m*M* of *p*-nitrophenol in 1 min. In all figures except Fig. 3, the activity of cells means the specific activity of 1 g of immobilized cells in granule. In Fig. 3, the activity means the activity of 1 g of granules.

ATP Concentration in Cells

ATP was extracted from free and immobilized cells by the treatment with DMSO *(13)*. The ATP concentration in immobilized cells was determined without preseparation of the cells from gel matrix but by the additional heat treatment of granules in the presence of DMSO at 80°C for 30–60 s. The ATP concentration was determined by the bioluminescence method with the aid of luciferin–luciferase reagent (Amersham Pharmacia Biotech, Uppsala, Sweden). ATP concentration in the extracts was calculated as it was described previously *(14)*.

Table 1
The Intracellular ATP Concentration
in Cells at Different Steps of Their Treatment

No.	Object of investigation	ATP, $\times 10^{-10}$ mole/mg of cells
1	Noncompetent free cells	2.74
2	Competent free cells	1.36
3	Competent free cells after transformation	0.23
4	Free transformants after 3 h of cultivation in the fresh medium	1.15
5	Immobilized competent cells after transformation	0.71
6	Immobilized transformants after 3 h of cultivation in fresh medium	3.19

Results and Discussion

First, it was necessary to understand how the different steps of immobilized transformants preparation could influence the intracellular ATP concentration. It is well known that the level of intracellular ATP is a remarkable indicator reporting on the cell response to different actions.

The results of ATP concentration monitoring in free cells before and after this step of their treatment with buffer containing various ions are represented in Table 1. These two steps are general for the preparation of both free and immobilized transformed cells. The data in Table 1 also clearly demonstrate the difference in intracellular ATP level of already obtained free and immobilized transformed cells. The data suggest that PVA-cryogel certainly plays the role of a cryoprotector and helps the competent cells to keep their viability under immobilized conditions.

The changes in ATP concentration in the immobilized transformants are presented in Fig. 1. The intracellular concentration of ATP in the cells strongly declined after 3 h of their cultivation in the fresh medium. We consider this tendency to be connected with the preparation of the cells to proliferation. Our later experiments confirmed this suggestion.

The typical plot of OPH activity of the immobilized cells transformed by double-stranded pOPf1 plasmid (100 ng/mL) as a function of cultivation time is also presented in Fig. 1. It demonstrates the principal possibility of transformation of competent cells by plasmid DNA and preservation of their ability to express proteins.

It should be noted that the process of transformation of free cells consists of several consecutive steps:

1. The diffusion and adsorption of DNA molecules on the surface of cells (reversible process); and
2. The penetration of genetic materials into the cells (irreversible process) during the heat-shock incubation of cells with DNA plasmid solution at 42°C.

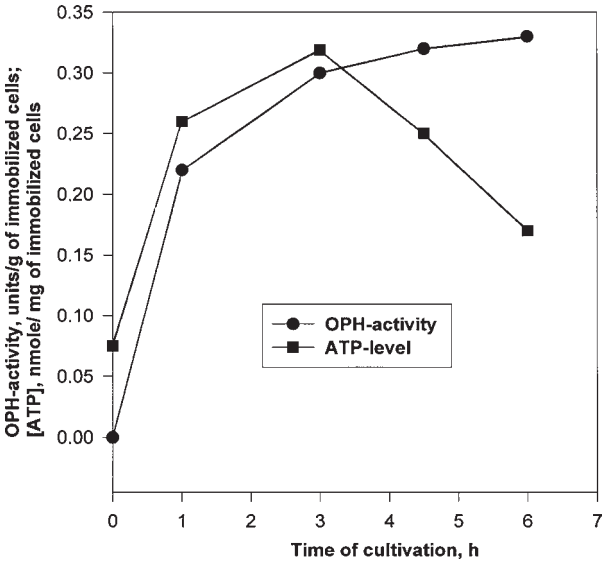

Fig. 1. The OPH-activity and ATP intracellular concentration in immobilized cells in the course of their cultivation.

Thus, the influence of the same steps on the transformation efficiency of the immobilized cells had to be checked.

To study the possible effect of the first stage of cell treatment on the transformation efficiency, the granules with the competent immobilized cells were incubated with DNA solution for 1, 2, and 3 h. However, no difference was observed (data not shown). So, we made a conclusion that the structure of PVA-cryogel did not create diffusion limitations for DNA molecules in this gene-transfer process.

The effect of heat-shock temperature on the process of transformation of immobilized cells is presented in Fig. 2. The incubation at 42°C is optimal for the transformation of free cells *(5)*. This temperature also appeared to be optimal among three values chosen for our experiment. It should be noted that the incubation of immobilized cells at 52°C results in a less than 50% decrease in transformation efficiency (estimated by activity levels after 2.5 h of cultivation) contrary to the free cells incapable of being transformed after heat-shock treatment under equivalent temperature conditions. These data also contribute to the supposition that PVA-cryogel matrix helps cells to retain their competent state longer.

The next stage of investigation was focused on optimization of the correlation between the quantity of cells inside the granules and the concentration of DNA plasmid solution used for transformation (Figs. 3 and 4).

The concentration of competent cells in granules used for transformation varied while the concentration of DNA solution was constant (Fig. 3). The concentration of cells in granules equal to 10% was shown to be the highest at the chosen DNA concentration when all accessible competent cells are saturated with DNA molecules and the further considerable

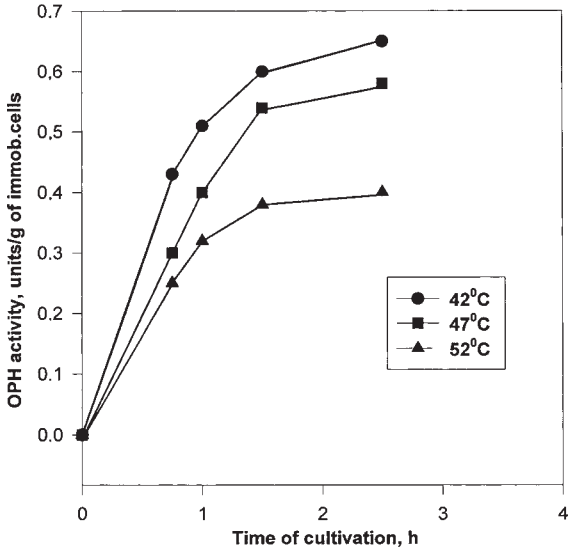

Fig. 2. Effect of heat-shock temperature on the OPH-activity of immobilized transformants.

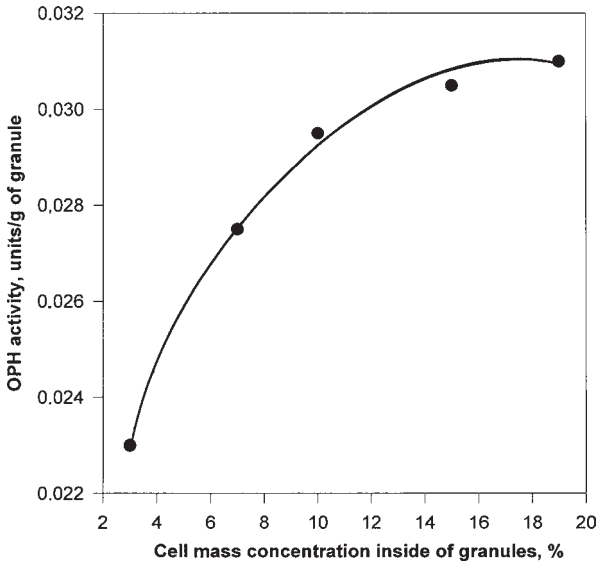

Fig. 3. Effect of mass concentration of cells within the biocatalyst on the specific activity of granules.

enhancement of activity of granules is not observed. At the same time, the highest real enzymatic activity per one cell in this experiment was observed in the case when the cell mass content in the granules was the lowest (Fig. 4).

Thus, the conditions of transformation are not favorable when the competent cells are closely packed together inside the granules, since such

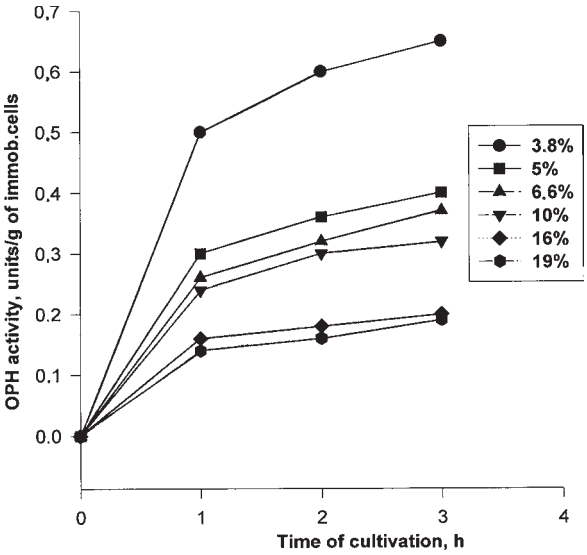

Fig. 4. Effect of cell-mass content in the granules of the biocatalyst on the OPH-activity of immobilized transformants.

package creates a diffusion barrier for DNA molecules and lowers the accessibility of the cells in general.

It was interesting to discover the correlation between the activity of immobilized transformants and the concentration of DNA molecules used for transformation. It was shown that there is the logarithmic relationship between the transformation efficiency of the immobilized competent cells and the plasmid concentration (Fig. 5), whereas it is linear for the free cells within the range of the chosen DNA concentrations. From our point of view, the possible explanation of this phenomenon is the fact that not all competent cells inside the PVA-cryogel granules participate in the transformation process but only those that are located close to the surface of granules.

In view of these findings, we consider that the preparation of the smallest granules for transformation is a real step to minimize diffusion limitations and to raise the transformation efficiency of the immobilized competent cells.

The last question to be answered in this study was the effect of the structure of DNA molecules on the transformation of immobilized cells.

The level of specific activity was compared for granules transformed by single-stranded and double-stranded pOPf1 plasmid DNA molecules. The data presented in Table 2 indicate that immobilization does not significantly interfere with cell transformation by different types of DNA molecules. In both cases, the transformation takes place. However, the transformation activity of single-stranded DNA molecules was about two times lower than that of a double-stranded plasmid DNA form.

The transformation of free cells in suspension demonstrated the same tendency. It should be noted that the immobilized cells did not significantly

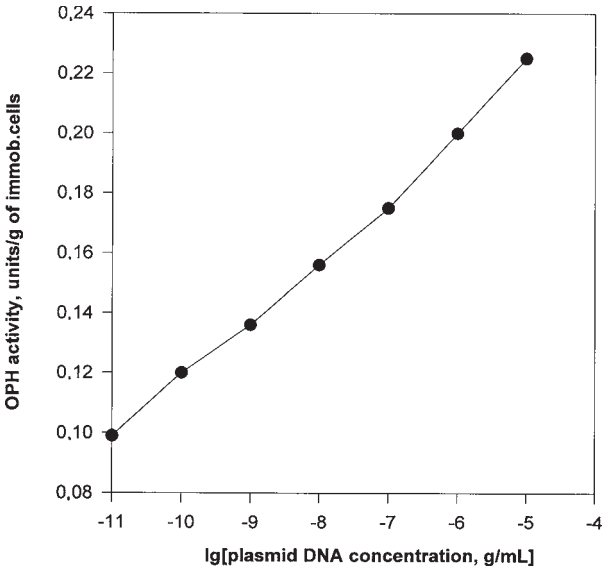

Fig. 5. Effect of plasmid DNA concentration on the activity of immobilized transformants.

Table 2
Comparison of Cell Transformation Efficiency by Single- and Double-Stranded Plasmid DNA Molecules

	Type of DNA molecules	
Samples of transformed cells	Single-stranded	Double-stranded
OPH-activity of immobilized cells after 10 h of cultivation, U/g of immobilized cells	0.24	0.43
OPH-activity of generated free cells in the medium with immobilized transformants after 10 h of their cultivation, U/g of cells	1.30	2.00

change their activity after 3 h of cultivation. The free transformed cells were accumulated in the cultural medium during cultivation of granules. The final activity of these free cells obtained as the next generation of immobilized transformants is presented in Table 2. The cells had the same level of activity as the transformants obtained by the free cells method.

Thus, our final experiments show that the immobilized transformants could be used, for example, as a persistent inoculum of a satisfactory quality. The storage of this inoculum in the form of granules with immobilized transformants could be organized in an ordinary refrigerator (4°C) in a buffer system with the addition of antibiotic and with regular substitution

of the medium at least every 3 mo without any significant change in their properties.

Conclusion

The present work for the first time shows the possibility of transformation of cells in immobilized state by plasmid DNA. It was found that immobilized competent *E. coli* cells entrapped in granules of PVA-cryogel preserved the ability to be transformed by both single- and double-stranded plasmid DNA molecules. Active protein synthesis was observed in transformed immobilized cells. The transformation efficiency of immobilized cells linearly depends on decimal logarithm of the plasmid DNA concentration. The investigation of properties of genetically engineered cells after their immobilization has unequivocal practical importance. The preserved competence of immobilized cells may help in the further development of new forms of competent cells with a prolonged storage viability. The question on the possibility of the immobilized genetically engineered cells to change their properties is also directly connected with the estimation of possible ecological risk of application of these biocatalysts in various environmental, agricultural or any other biotechnological fields.

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