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Investigation of feasibility of gene transfer in *Trichosporon cutaneum* yeast strain R57

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ABSTRACT

The experiments on the genetic transformation of *Trichosporon cutaneum* R57 protoplasts were performed. In the present work we report the development of a system for chromosomal transformation in *Trichosporon cutaneum* R57. The carried out mutagenesis gave as a result the obtaining of suitable for our purpose auxotrophic mutants of the studied yeast strain. The isolated transformants were with increased resistance to phenol as compared to the recipient culture under selective conditions. The successful fulfillment of an intra-species protoplast fussion as well as an inter-generic protoplast fussion between *Trichosporon cutaneum* and *Candida tropicalis* strains is presented.

Key words: *Trichosporon cutaneum* R57, mutagenesis, genetic transformation, protoplast fusion

Introduction

Significant efforts have been made to accomplish an efficient DNA transformation system and investigate the genetic organization and characteristics of yeasts of the genus *Trichosporon* (Diez-Ruiz B., 2002). Glumoff et al. (1989) manage to create such a system on the basis of plasmids carrying dominant selectable marker - hygromycin (Glumoff et al., 1989). Another transformation system for *Trichosporon cutaneum* has based on the auxotrophic markers and techniques for induction, isolation and characterization of the mutants (Ochner et al., 1991).

Gene of phenol hydroxylase is isolated, cloned, sequenced and expressed in *E. coli* cells under the control of *tac* promoter in an attempt to create expression vectors. Several auxotrophic strains of *Trichosporon adeninovorans* have been isolated by UV-radiation or by the use of N-methyl-N- nitro-N-nitrosoguanidine (Samsonova et al., 1989). The different biochemically defined mutants of *T. adeninovorans* gave appropriate chromosomal markers for future genetic studies of these yeasts. A transformation system based on *T. adeninovorans* lysine - auxotrophic mutants, complementary to cloned genes of the lysine biosynthesis of *Saccharomices cerevisiae* has also been developed (Butner et al., 1990).

The possibility to study yeast without proven sexual process, raised after the detection of the method for protoplast fusion. This method is described as somatic hybridization, cell fusion or spheroplast fusion. There is no universal method described for the application of this method in microbiology. A large number of intact protoplasts obtaining is crucial for the successful protoplast fusion (Freeman, 1983; Ferenczy, 1981).

Various methods are used for the preparation of protoplasts (Peberdy, 1980; Delgado et al., 1981; Rivera et al., 2014). The most commonly used method is the enzymatic lysis of the cell wall (Kevei, 1981; Hamlin et al., 1981; Diez-Ruiz, 2002). A crucial condition is the growth phase of the culture. The hybridization of yeast strains might be conducted by protoplast fusion of the same species (intra-species hybridization), but also between species taxonomically distant (inter- species and inter-generic hybridization).

The first protoplast fusion is performed in *Geotrichum candidum*, using strains marked with auxotrophic mutations. The most used confluent agent for this procedure is polyethylene glycol (PEG molecular weight 4000 - 8000). The conditions under which appears coacervating effect of PEG are similar for different types of yeast (Corner et al., 1989; Horitsu et al., 1989). The ability of protoplasts to regenerate the cell wall is an important condition for

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successful protoplast fusion. The selection of the obtained protoplast might be performed on a minimal medium with suitable additives.

The successfully implemented intra-species protoplast fusion between *Candida tropicalis* auxotrophic mutant and phe$^-$ mutant leaded to isolation of fuzants able to digest >/= 4 g/l phenol (Chang et al., 1998). The results of the limited research on genetics of the *Trichosporon cutaneum* strains show that they posses a different ploidyty, but nevertheless they are fused to a high degree. This condition, along with methods of transformation and cloning techniques, provides a basis for efficient genetic manipulation in *Trichosporon cutaneum*, despite the absence of sexual reproduction in the organism (Glumoff et al., 1989; Ochsner et al., 1991).

An intra-species protoplast fusion was performed with species of *Trichosporon cutaneum* as well. According to the literature, the formation takes place of transition diploids which segregate spontaneously and often provide generation of combinations of markers, different from the parent (Sienko et al., 1992).

Materials and Methods

Strains, media and culture conditions

In the research process were used the strain *Trichosporon cutaneum* R 57 registered in the National Bank of Industrial Microorganisms and Cell Cultures under N 2414/1994, and the strain of *Candida tropicalis* Tul 1. The complete and synthetic broth media were used for yeast cultivation, fusion, transformation and protoplast regeneration. Glucose, phenol and acetate in appropriate concentrations were used as carbon sources. The main parameters of the cultivation are: T = 28-30°C; aeration at 180-200 rpm/min on a rotary shaker.

Isolation of chromosomal DNA

Trichosporon cutaneum R57 cells were cultured until a middle logarithmic phase of growth in a liquid salts medium. The first step of chromosomal DNA isolation followed methods for the protoplasts preparation. The DNA was isolated from the obtained protoplasts (Holm et al., 1986; Hoffman et al., 1987).

Protoplast transformation

1 – 10 μg DNA were added to 0.1 ml of the protoplast suspension. After 15 min incubation at 22°C to the suspension was added 1 ml of PEG buffer while stirring carefully and the suspension was incubated again at 30°C for 20-30 minutes. Part of the suspension (0.2-1 ml) was mixed with 5 ml top - agar (45°C) comprising a selection marker and was transferred on Petry dishes with pre-solidified regeneration agar (Glumoff et al., 1989). The plates were incubated for 2 to 5 days.

Protoplast fusion

The resulting protoplasts were washed and resuspended in a solution containing 1.2 M sorbitol and Ca/Tris buffer (pH = 7.5). The two parental protoplasts types were mixed in a 1:1 ratio. 10 volumes of 20% solution of PEG 6000, and Ca/Tris buffer were added to the mixture (0.1 ml) (Chang et.al., 1995; Sienko et.al., 1992). The suspension was mixed with 8 ml selective top agar at 45°C and quickly transferred to Petri dishes containing solid regeneration medium after 15 minutes incubation at 30°C. The plates were incubated for 4 - 6 days at 30°C. The unused parental protoplasts were grown on a non-selective regeneration agar in order to establish the efficiency of the regeneration process.

Results

Obtaining mutants of Trichosporon cutaneum R57

In the process of searching for a suitable method for producing and selection of *Trichosporon cutaneum* R57 mutants we opted for mutagenesis with N-nitro-N-methyl-N-nitrosoguanidine, which adapted to the specific characteristics of the strain and our genetic tags. The survival of the culture after treatment with nitrosoguanidine was in the range of 1.2%.

The mutagenesis with nitrosoguanidine showed low frequencies of obtaining mutants (<0.01%). The frequency was increased by using the antibiotic nystatin. A key factor for the mutants obtaining turned to be the time of incubation in minimal medium (for auxotrophs) or a medium with a low concentration of phenol (for those with reduced resistance phenol) prior to the treatment with nystatin.

Combining all the optimal parameters, we have achieved increases in the efficiency of mutagenesis (~ 0.1%). As a result of mutagenesis and selection medium containing no carbon source (YNB w / o AA) were isolated 20 auxotrophic mutants in different trophic factors.

Our observations showed that their auxotrophy remains after repeated passaging, but most have demonstrated significant morphological changes that ultimately lead to their loss. As a result, we kept only 5 mutants for the following experiments. Phenotypically they are divided as

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follows: 2-adenine$^-$; 1-arginine$^-$; 1-adenine$^-$, 1-arginine$^-$; 1-adenine$^-$ and valine$^-$.

In the established pattern of mutagenesis were obtained mutants more sensitive to phenol as compared to the parent strain and mutants capable of degrading higher than 1 g/l phenol included in the culture medium. The total number of the obtained mutants of these groups is 42. Figure 1 shows a number of mutants obtained, divided by their resistance to the presence of phenol in the medium. From the data shown it can be seen that 38 of the selected mutants are with reduced resistance to phenol as compared to the parent strain, but only four of them exhibits a higher resistance. Unfortunately mutant MR (resistant to 1.4 g/l phenol), proved to be unstable and was not used for the next. An interesting fact is that our selected mutants with altered resistance to phenol retain morphological characteristics of the parent strain and with few exceptions are stable and do not revert.

To perform the experiments for interspecies protoplast fusion were used two of the isolated *T. cutaneum* R57 (*bio-, thy-)* mutants: mutant N3 (*arg-, bio-, thy-)* mutant and N2 (*ade-, bio-, thy-),* capable to degrade respectively 0.3 g/l or 0.5 g/l phenol in the culture medium.

In the study of the feasibility of genetic transformation have been used mutants S1 (resistant to 0.3 g/l) and A1 (*phe-, ade-, bio-, thy-).* For studies related to peculiarities of the key enzymes involved in the phenolic biodegradation by *Trichosporon cutaneum* R57 were selected mutants 2R and 4R resistant to 1.2 g/l and 23S mutant - unable to grow at concentrations of phenol higher than 0.6 g/l. Mutants involved in the different experiments were selected according to the type of owned by them genetic markers, as well as their stable inheritance.

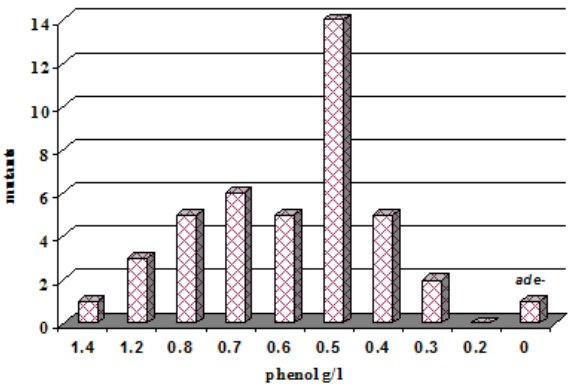

Figure 1. *Distribution of mutants of Trichosporon cutaneum R57 by resistance to various concentrations of phenol in the culture medium.*

Protoplast fusion in Trichosporon cutaneum R57

In the experiments for intraspecies protoplast fusion of the mutants the obtained efficiency was about 35 - 40%, which is typical for the parent strain. The efficiency of the regeneration of protoplasts was 60% to 50%. In result of the subsequent selection were obtained 10 fuzants where was not detected any adenine or arginine auxotrophy and the resistance (1 g/l) to phenol was recovered. The Table 1. reflects the genetic characteristics of the strains involved in the process and the frequency of the protoplast fusion. Our observations showed that the resulting fuzants retained their morphological characteristics, but were genetically unstable and segregate to the output format. Interesting results were obtained from the experiments for the realization of intergeneric fusion.

Table 1. *Protoplast fusion in Trichosporon cutaneum R57.*

Strains	Genetic markers	Frequency of mutants' reversion	Fusion frequency	Frequency of fusants' reversion
		Wild type		
R 57	*bio$^-$, thy$^-$*, 1.0g/l phenol			
Tul 1	prototroph, 0.5 g/l phenol			
N3	*arg,$^-$bio$^-$, thy$^-$* 0.3g/l phenol	$< 10^{-6}$		
N2	*ade,$^-$bio$^-$, thy$^-$* 0.5g/l phenol	$< 10^{-7}$		
		Fusants		
N2 x N3	prototroph 1.0g/l phenol		1×10^{-4}	1×10^{-3}
R 57 x Tul 1	F1 prototroph 1.0g/l phenol			
	F1,2 prototroph 1.2g/l phenol		3×10^{-5}	1×10^{-3}

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As an object of the mating strain was selected strain *Candida tropicalis* Tul1 which is prototroph and is able to grow on a medium containing up to 0.5 g/l phenol. The efficiency of obtaining protoplasts in these experiments ranged from 35-40% for Trichosporon cutaneum R57 and 50 - 60% of a Candida tropicalis Tul1.

Protoplast transformation of strain Trichosporon cutaneum R57

Total DNA isolated from the strain *Trichosporon cutaneum* R57 was used to transform protoplasts of mutants with impaired resistance to phenol. The sensitivity to phenol was used as a selectable genetic marker. In the Table 2 are presented data characterizing the process of chromosomal transformation of the studied strain.

Table 2. *Chromosomal DNA protoplast transformation of Trichosporon cutaneum R57.*

Strains	Frequency of transformation	Efficiency of transformation
S1 *bio*$^-$, *thy*$^-$,0.3 g/l	1.9x10^{-2}	75 transformants/µg DNA
A1 *bio*$^-$, *thy*$^-$, *ade*$^-$, *phe*$^-$	2.1x10^{-2}	200 transformants/µg DNA

In the process of transformation were used protoplasts of the mutant S1, which is resistant to 0.3 g/l phenol. Initially were obtained 152 colonies growing on rich medium (YEP) containing 0.5 g/l phenol. By subsequent selection were isolated 32 stable transformants growing on a mineral medium (MM) comprising different phenol concentration as the sole carbon and energy source. Isolated transformants were distributed as follows: 26 - resistant to 0.5 g/l phenol and 6 - resistant 1.0 g/l phenol.

In the transformation of protoplasts of a mutant A1, sensitive to the presence of phenol in the medium and ade$^-$, were isolated 500 colonies grown on rich medium containing 0.5 g/l phenol. Of these were isolated 20 stable transformants. 8 of them are resistant to 1 g/l phenol contained in a rich medium (YEP), and only 5 of these grow in mineral medium containing 1 g/l phenol as the sole carbon and energy source.

Discussion

Implementation of genetic transfer in Trichosporon cutaneum R57

The feasibility of molecular genetic manipulation with the genome of studied *Trichosporon cutaneum* R57 strain would extend our views on the overall biology of this microorganism. *Para*-sexual studies of the species *Trichosporon cutaneum* were conducted in a limited number of strains (Reiser et al., 1994; Ochsner et al., 1991). The absence of strains with appropriate genetic markers is a barrier in the implementation of gene transfer by protoplast fusion or genetic transformation.

As a pre-stage to the completion of these processes, we describe a technique for the preparation and characterization of auxotrophic mutants, and mutants in which the resistance to phenol was changed. The purpose was to make these mutants suitable recipients in gene transfer processes. Upon mutants obtaining, in order to increase the efficiency of the process the culture was treated with the antibiotic nystatin. It is known that this antibiotic is capable of killing selectively growing yeast cells, as it prevents the formation of the cell wall. In the same conditions, mutant cells do not grow and manage to survive.

In our experiments were obtained mainly *ade*$^-$ and *arg*$^-$ mutants. These results may be due to the different sensitivity of these mutants to Nystatin, features the initial phenotype that affects the initial screening, inability to acquire sufficient supplements to ensure growth or low frequency with which these mutants arise at this type of mutagenic treatment.

The second type of isolated mutants was with altered tolerance to the presence of phenol in the culture medium. Experiments with them aim to show the possibility for application of genetic methods to influence the ability of the studied strain to grow and develop in an environment with a single carbon source phenol. From the data shown in Figure 1 could be concluded that most of the obtained mutants (38) are with lowered but not totally disappeared resistance. Several mutants with slightly increased resistance were also received. Mutants with altered characteristics with respect to the utilization of phenol were used in the following experiments to assay the activity of phenol - degrading enzymes.

The valuable qualities of the number of *Trichosporon cutaneum* strains maid them a subject of genetic development by means of classical and molecular genetic methods. One aspect of these studies is the establishment of an efficient transformation system. The successful use of *Trichosporon cutaneum* as a recipient organism for the expression of foreign genes is described by Clumoff et al., (1989) and Reiser et.al., (1994) . Both studies reported the realization of the plasmid transformation, and expression of cloned genes to

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other organisms in this yeast specie.

The aim of our experiments on the transformation of *Trichosporon cutaneum* R57 was different. We set ourselves the task to check whether it is possible transformation within the strain, but with chromosomal DNA. As evidenced by the reported results, we were able to develop a system for chromosomal transformation in *Trichosporon cutaneum* R57 (Table 2). Similar transformation experiments have not been reported by other authors (Gietz & Woods, 2001).

Conclusion

Mutants of *Trichosporon cutaneum* strain R57 were isolated, bearing genetic markers for auxotrophy by adenine, arginine and valine, as well as mutants with an altered resistance to phenol.

Fuzants with increased resistance to phenol were received by intraspecies and interspecies protoplast fusion with *Trichosporon cutaneum* R57.

Chromosomal protoplast transformation of *Trichosporon cutaneum* R57 was first performed. Isolated transformants are with increased resistance to phenol as compared to the recipient culture under selective conditions.

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