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FERMENTATIVE ACTIVITY OF SOMATIC HYBRIDS OF *SACCHAROMYCES CEREVISIAE* AND *CANDIDA SHEHATAE* OR *PACHYSOLEN TANNOPHILUS*

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ABSTRACT

The aim of the research was to obtain interspecific hybrids of distilling yeast and xylose-fermenting yeast and to assess their fermentative activity in media containing xylose. Distilling yeast *Saccharomyces cerevisiae* D₄₃ and xylose-fermenting yeast *Candida shehatae* ATCC 58779, *Pachysolen tannophilus* ATCC 32691 and *Pachysolen tannophilus* ATCC 60392 were used. Medium containing xylose as the sole carbon source was used for selecting the interspecific hybrids. Incubation temperature (42°C) and crystal violet (0.01%) were used as additional selection factors for hybrids of the yeast *Candida shehatae* ATCC 58779 and *Pachysolen tannophilus* ATCC 32691 or *Pachysolen tannophilus* ATCC 60392, respectively.

As a result of the fusion of protoplasts in polyethylene glycol, 7 stable mutants of *Saccharomyces cerevisiae* D₄₃ and *Candida shehatae* ATCC 58779 and 3 stable mutants of *Saccharomyces cerevisiae* D₄₃ and *Pachysolen tannophilus* ATCC 60392 were obtained. The CD43-7, CD43-9 and Pt60D43-1 mutants efficiently fermented both glucose and xylose. Electrophoretic analysis of the products of PCR obtained with the use of a microsatellite primer (GTG)₅ did not unequivocally confirm recombination of DNA of parental strains.

Key words: distilling, distillery yeast, protoplast fusion, xylose fermentation

INTRODUCTION

High prices of fuels derived from crude oil and depletion of fossil fuel reserves result in growing interest in alternative energy sources. One of such ecological sources of energy is ethanol. In order to make its price competitive with the price of traditional fuels, it is necessary to use modern fermentation systems and to use cheap substrates such as biomass, agricultural, industrial and municipal wastes or forestry residues [26]. Fermentation of xylose - the main component of hemicelluloses - is one of the main factors determining profitability of production of ethanol from lignocellulose. The analysis of fermentative ability of yeasts shows that almost half of the known species can assimilate D-xylose, but cannot ferment it. Such yeasts as *Pichia*, *Candida*, *Pachysolen*, *Kluyveromyces*, *Brettanomyces*, *Debaryomyces*, *Schizaosaccharomyces* and *Clavispora* are characterised by ability to both assimilate and ferment xylose [10]. It is still impossible to design an effective technological process with their use due to low ethanol yield obtained by microorganisms fermenting xylose, their sensitivity to higher ethanol concentration, preferential fermentation of glucose in mixture of sugars contained in lignocellulose and difficulties in optimising physicochemical parameters of fermentation and the necessity of maintaining a controlled oxidation of the medium. Moreover, the microorganisms fermenting xylose are sensitive to inhibitors present in hydrolysates, both those appearing during hydrolysis (e.g. acetic acid, terpenes, alcohols, aromatic compounds including tannins, products of sugars degradation: furfural, 5-hydroxymethylfurfural, laevulinic acid, formic acid and humic substances), those coming from the equipment (mainly metals: chromium, cuprum, iron, nickel) and those added during the process (e.g. SO₂) [7, 16].

In case of the yeast *Saccharomyces cerevisiae*, neither assimilation nor fermentation of xylose is possible due to low activity of the enzymes of xylose reductase and xylitol dehydrogenase and the lack of balance in the redox system NAD/NADH. As the yeast *Saccharomyces cerevisiae* is one of the best microorganisms producing ethanol, there are attempts at constructing such *Saccharomyces cerevisiae* recombinants that could ferment hexoses, pentoses and also disaccharides, such as e.g. cellobiose [8, 17].

The results obtained in numerous studies seem to confirm the significance of protoplast fusion as a method of improving industrial yeast strains, despite difficulties in introducing specific genes and low stability of recombinants, esp. of taxonomically different species [14, 25].

The present study is an attempt at using interspecific protoplast fusion to add conversion of D-xylose to ethanol to the fermenting abilities of distillery yeast *Saccharomyces cerevisiae*.

THE AIM OF THE RESEARCH

The aim of the research was to obtain interspecific hybrids of distillery yeasts *Saccharomyces cerevisiae* D₄₃ and *Candida shehatae* ATCC 58779, *Pachysolen tannophilus* ATCC 32691 or *Pachysolen tannophilus* ATCC 60392 that could effectively ferment xylose.

The following activities were performed:

- choosing markers enabling selection of hybrids,
- obtaining interspecific hybrids by protoplast fusion,
- confirming DNA recombination by comparative analysis of genomic DNA of parental strains and hybrids,
- assessing the fermentative activity of selected stable hybrids in media containing xylose.

MATERIALS AND METHODS

Biological material

Distillery yeast *Saccharomyces cerevisiae* D₄₃ from the Collection of Cultures of the Department of Food Storage and Technology (Agricultural University of Wroclaw) and xylose fermenting yeasts *Candida shehatae* ATCC 58779, *Pachysolen tannophilus* ATCC 32691 and *Pachysolen tannophilus* ATCC 60392 were used in the present study.

Selection of markers

Selection of markers differentiating parental strains was performed on solid media YPX with xylose (20 g/dm³) as the sole carbon source. Moreover, the ability of the strains used in the study to grow on solid medium YPG with cycloheximide (0.1, 0.01 and 0.001%) [3] and with crystal violet (0.1, 0.01 and 0.001%) [12] and at 30°C and 42°C was examined.

Formation of protoplasts

Protoplasts were formed with a lytic enzyme from *Trichoderma harzianum* (Novozym™ 234 SIGMA 2mg/cm³ 0.6 M KCl, incubation up to 1h at 30°C). 0.6 M KCl was used as osmotic stabiliser [5, 13]. The rate of protoplasts formation [%] and regeneration of the protoplasts [%] were determined.

The fusion of protoplasts of distillery and selected xylose-fermenting yeasts was induced using a solution of polyethylene glycol (PEG 6000) in 10mM CaCl₂ (60 min., 30°C) [5, 6]. The frequency of fusion was calculated according to Sakai et al. [20].

The obtained hybrids were transferred to YM slants and kept at 4°C. After 4, 8, 12 and 24 weeks of storing, the stability of hybrids was examined by controlling their growth in selection medium.

Assessment of fermentative activity

Initial assessment of the ability of the hybrids to ferment xylose was conducted in media containing xylose (YPX) in tubes with Durham tube at 30°C [24]. Approximate content [% vol.] of CO₂ in Durham tube after 48, 120 and 168 hours of fermentation was assessed, taking full packing of the tube with gas as 100%.

Fermentative tests with somatic hybrids were conducted in Einhorn tubes in media with xylose (YPX) and glucose (YPG) at the temperature of 30°C. The content of CO₂ [cm³] secreted in the Einhorn tube was compared after 7 days of fermentation.

Analysis of genomic DNA of parental and hybrid strains

Isolation of DNA from the cells of parental and hybrid strains was performed using the Rose et al. method [19] in Skała modification [22] and Zymolase 5000 (Seikagaku Kogyo Co., Ltd) at the concentration of 20 mg/cm³ [21]. Polymerase chain reaction (PCR) using thermostable Taq DNA of polymerase (Core KIT 01223, QIAGEN) in Peltier Thermal Cycler The PTC - 200 DNA Engine™ MJ Research, Inc. thermocycler was used for duplicating matrix DNA. Amplification of DNA of parental and somatic hybrids' strains was performed using microsatellite primer (GTG)₅ (ARK SCIENTIFIC) [1, 2, 11] and a thermal profile of the reaction: 40 cycles - DNA denaturation (93°C, 20 s), primer binding (50°C, 1 min), DNA synthesis (72°C, 20 s).

The products of PRC reaction were analysed by electrophoresis, using 1.5% agarose gel with ethidium bromide and standard of mass (100 bp DNA Ladder Plus, Promega). DNA separation on agarose gel was documented using a SONY TV recording set in UV light.

RESULTS AND DISCUSSION

Selection of markers for identification of hybrids

To properly isolate colonies of hybrids from the mixture of parental and hybrid strains, each of the parental strains was assigned two differentiating features (markers), which, as a result of fusion, are completed by the hybrid.

The ability of *Saccharomyces cerevisiae* D43, *Candida shehatae* ATCC 58779, *Pachysolen tannophilus* ATCC 60392 to grow on media with glucose and xylose was compared.

The presence of xylose as the sole carbon source in the medium was one of the features of a selection marker (Table 1).

Table 1. The ability to consume glucose and xylose by the strains of yeasts used in the research

Yeast strain	carbon source	
	glucose	xylose
<i>Saccharomyces cerevisiae</i> D43	+	–
<i>Candida shehatae</i> ATCC 58779	+	+
<i>Pachysolen tannophilus</i> ATCC 32691	+	+
<i>Pachysolen tannophilus</i> ATCC 60392	+	+

(+) –growth, (–) – lack of growth

In the search for the second marker, varying tolerance of the strains to higher temperature was taken into account. [Table 2](#) presents a comparison of growing ability of the strains used in the present study in YPG media at 30°C and 42°C. The ability to grow at higher temperature (42°C) was observed in the strains *Saccharomyces cerevisiae* D43, *Pachysolen tannophilus* ATCC 32691 and *Pachysolen tannophilus* ATCC 60392 whereas the yeast *Candida shehatae* ATCC 58779 were more sensitive to the temperature of incubation. This feature was selected as the second selection marker for differentiating the hybrids of *Saccharomyces cerevisiae* D43 and *Candida shehatae* ATCC 58779.

Table 2. The growing ability of the strains of yeasts used in the research in YPG at 30°C and 42°C

Yeast strain	30° C	42° C
<i>Saccharomyces cerevisiae</i> D43	+	+
<i>Candida shehatae</i> ATCC 58779	+	–
<i>Pachysolen tannophilus</i> ATCC 32691	+	+
<i>Pachysolen tannophilus</i> ATCC 60392	+	+

(+) – growth, (–) – lack of growth

Moreover, the resistance of the strains *Saccharomyces cerevisiae* D43, *Pachysolen tannophilus* ATCC 32691 and *Pachysolen tannophilus* ATCC 60392 to cycloheximide, an antibiotic typically used for inhibiting the growth, and to crystal violet, a dye used in bacteriology as an important selective and differentiating factor. Lin and Fung [12] examined inhibiting influence of 101 organic dyes on growing abilities of 33 strains of yeasts. Crystal violet, even at extremely low concentration, was an inhibitor of all strains under research except *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Saccharomyces glbosus* and *Candida lipolitica*. The yeasts *Saccharomyces cerevisiae* D43 and *Pachysolen tannophilus* were tested on media with various concentrations of cycloheximide and crystal violet ([Table 3](#)).

Table 3. The growing ability of the strains of yeasts used in the research in YPG media with cycloheximide and crystal violet

Yeast strain	YPG+ cycloheximide			YPG+ crystal violet		
	0.1%	0.01%	0.001%	0.1%	0.01%	0.001%
<i>Saccharomyces cerevisiae</i> D43	–	–	–	–	+	+
<i>Pachysolen tannophilus</i> ATCC 32691	–	–	–	–	–	–
<i>Pachysolen tannophilus</i> ATCC 60392	–	–	–	–	–	–

(+) – growth, (–) – lack of growth

Neither *Saccharomyces cerevisiae* D43 nor any strain of *Pachysolen tannophilus* showed growing ability at even the lowest concentration of cycloheximide (0.001%) in the medium. Moreover, the two strains of did not grow in the presence of crystal violet, whereas in case of *Saccharomyces cerevisiae* D43, growth was observed on media with 0.01% and 0.001% of the dye. For these partner strains, the ability to grow in the presence of crystal violet (0.01%) was selected as the second marker.

Based on the results obtained in the study, a medium with xylose as the sole carbon source, on which only *Saccharomyces cerevisiae* D43 did not grow, was chosen for the selection of interspecific hybrids. In order to select hybrids of *Saccharomyces cerevisiae* D43 and *Candida shehatae* ATCC 58779, the media were incubated at 42°C (no growth of the yeast *Candida shehatae* ATCC 58779). The agent differentiating the hybrids of *Saccharomyces cerevisiae* D43 and *Pachysolen tannophilus* ATCC 32691 or *Pachysolen tannophilus* ATCC 60392 was crystal violet (0.01%). No growth of *Pachysolen tannophilus* ATCC 32691 and *Pachysolen tannophilus* ATCC 60392 was observed in its presence.

Obtaining, stability and assessment of fermentative ability of interspecific hybrids

The first objective was to obtain protoplasts of parental strains. The rate of protoplasts formation and the protoplasts' ability to regenerate the cell walls in solid medium were assessed. The rate of protoplasts formation and the rate of protoplasts' regeneration are presented in [Table 4](#).

Table 4. Protoplasts formation and regeneration of protoplasts of the yeast strains used in the research

Yeast strain	The rate of protoplasts formation [%]	The rate of protoplasts' regeneration [%]
<i>Saccharomyces cerevisiae</i> D43	91.9	67.3
<i>Candida shehatae</i> ATCC 58779	98.8	61.3
<i>Pachysolen tannophilus</i> ATCC 32691	95.4	48.2
<i>Pachysolen tannophilus</i> ATCC 60392	98.1	66.4

The rate of protoplasts formation of the yeast cells in the set conditions exceeded 90% and was 91.9% in case of *Saccharomyces cerevisiae* D43 and up to 98.8% in case of *Candida shehatae* ATCC 58779. It should be noticed that such a high rate of protoplasts formation of all xylose fermenting yeasts was noted as soon as after 20 minutes of incubation in lytic solution, whereas in case of *Saccharomyces cerevisiae* D43 the process lasted ca. 1 h. Javadekar et al. [9] reported a characteristic susceptibility of yeasts *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* to Novozyme 234. After incubation of these strains in the presence of dithiothreitol and 1 mg/cm³ concentration of Novozyme, they obtained 100% of protoplasts in 15 minutes. Obtaining the same results of protoplasts formation of the yeast *Saccharomyces cerevisiae* in the conditions presented above required four-times longer time of reaction. The rate of protoplasts regeneration varied from 48.2% to 67.3%. *Saccharomyces cerevisiae* D43 was characterised by the best ability to regenerate the cell wall.

[Table 5](#) presents the results of the protoplasts fusion of distillery yeasts and xylose-fermenting yeasts. 18 hybrids of *Saccharomyces cerevisiae* D43 and *Candida shehatae* ATCC 58779, 1 hybrid of *Saccharomyces cerevisiae* D43 and *Pachysolen tannophilus* ATCC 32691 and 10 hybrids of *Saccharomyces cerevisiae* D43 and *Pachysolen tannophilus* ATCC 60392 were isolated.

Table 5. The results of the fusion of protoplasts of distilling and xylose-fermenting yeasts

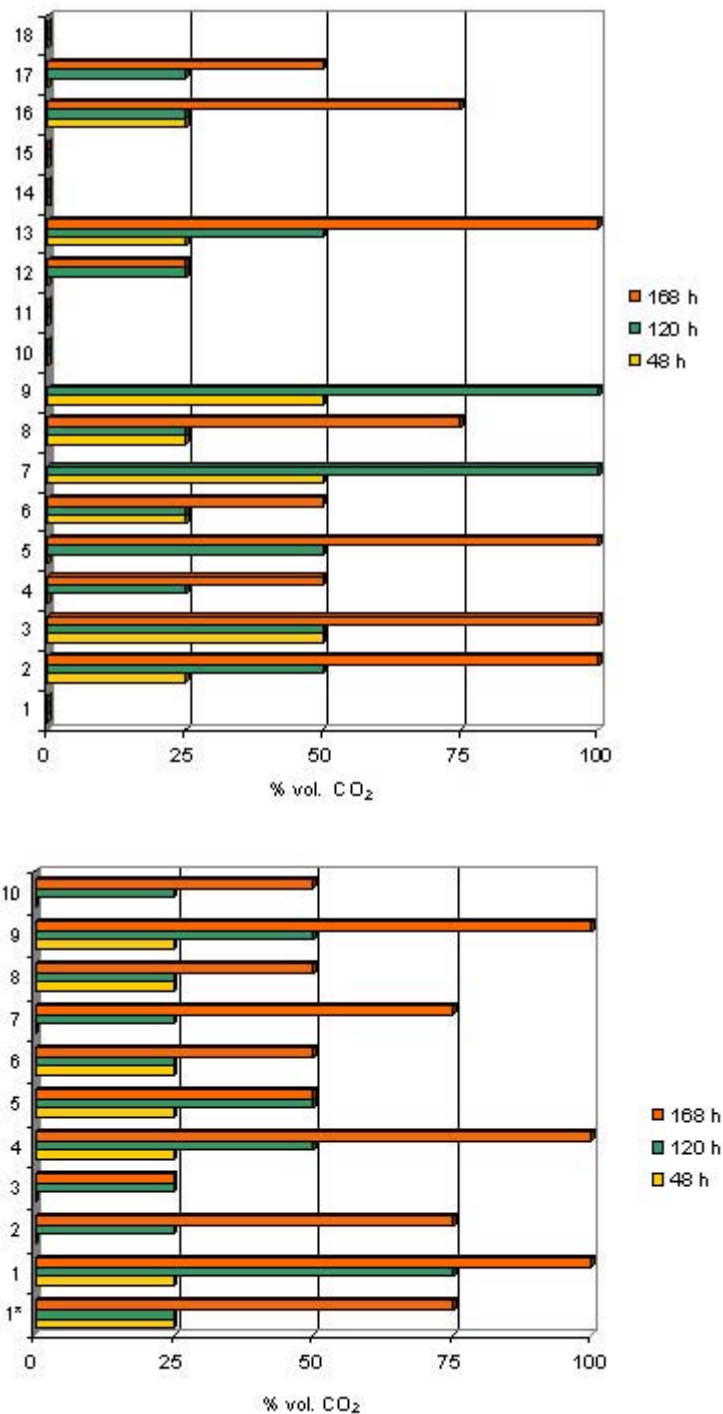
Parental strains	Symbols of hybrids	Number of hybrids	The frequency of fusion
<i>Saccharomyces cerevisiae</i> D43 <i>Candida shehatae</i> ATCC 58779	CD43-1÷ CD43-18	18	2.01×10^{-5}
<i>Saccharomyces cerevisiae</i> D43 <i>Pachysolen tannophilus</i> ATCC 32691	Pt32D43-1	1	1.86×10^{-6}
<i>Saccharomyces cerevisiae</i> D43 <i>Pachysolen tannophilus</i> ATCC 60392	Pt60D43-1÷ Pt60D43-10	10	1.26×10^{-5}

The frequency of fusion did not depend on diverse conditions of the selection and was from 1.86×10^{-6} in case of the fusion of *Saccharomyces cerevisiae* D43 and *Pachysolen tannophilus* ATCC 32691 to 1.26×10^{-5} in case of *Saccharomyces cerevisiae* D43 and *Pachysolen tannophilus* ATCC 60392. The number of hybrids obtained was higher at the temperature of 42°C used as a marker, which may imply that the physical selection factor was not as strong as crystal violet, the chemical factor.

From technological point of view, the assessment of the ability of the hybrids to ferment xylose was very important. Fermentation tests, during which the dynamics of CO₂ production with Durham tubes was assessed, were performed.

6 out of 18 the hybrids of *Saccharomyces cerevisiae* D43 and *Candida shehatae* ATCC 58779 – CD43-1, CD43-10, CD43-11, CD43-14, CD43-15 and CD43-18 did not show an ability to convert xylose to ethanol ([Fig. 1](#)). The hybrids CD43-7 and CD43-9 were characterised by the best converting ability, they finished the fermentation after 120h. CD43-12 was characterised by the worst fermenting ability – only 25% of total CO₂ was collected in a Durham tube after 168h.

Fig. 1. Fermentative ability of the hybrids CD43, Pt32D43-1 and Pt60D43 during fermentation of xylose (% vol. of CO₂ produced in Durham tubes)



When compared with the other hybrids of yeasts, the hybrids of both strains of *Pachysolen tannophilus* were characterised by low dynamics of fermenting xylose. Only 3 Pt60D43 hybrids, i.e. Pt60D43-1, Pt60D43-4 and Pt60D43-9 finished fermentation after 168h. The Pt60D43-3 hybrid was characterised by the lowest fermenting ability.

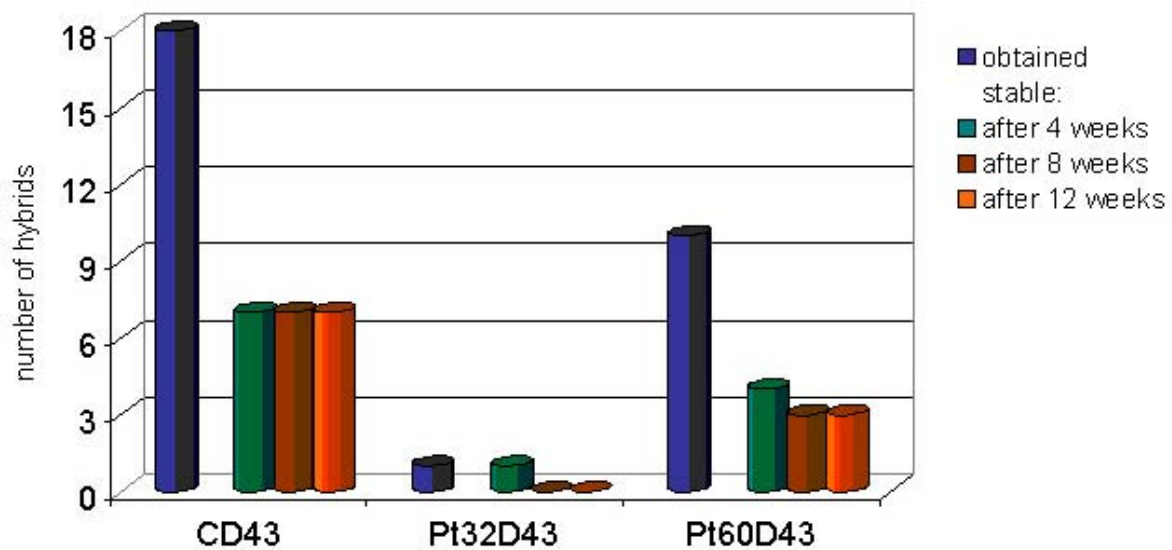
The aim of this stage of selection was to choose the strains of hybrids that can ferment xylose. 6 of the obtained hybrids were rejected, as they did not ferment xylose. They were the hybrids of *Candida shehatae* ATCC 58779 and *Saccharomyces cerevisiae* D43: CD43-1, CD43-10, CD43-11, CD43-14, CD43-15 and CD43-18.

As the measurements of fermenting ability are not sufficiently accurate, all the hybrids that could ferment xylose had to be examined in further tests.

The products of fusion should be stable during storage and should not revert to their primary forms, which may occur when taxonomically different species of yeasts undergo the process of fusion. For this reason, the stability of new features after 4, 8, 12 and 24 weeks of storing was examined by assessing the growing ability of the hybrids.

Almost 60% of the hybrids of the yeasts *Candida shehatae* ATCC 58779 and *Saccharomyces cerevisiae* D43 lost the features received from parental strains in the first 4 weeks. However, the other hybrids were characterised by stability of the features that were received (Fig. 2). All hybrids of *Pachysolen tannophilus* ATCC 32691 and *Saccharomyces cerevisiae* D43 lost the features received from parental strains after 8 weeks of storing. The hybrids of the other strain of *Pachysolen tannophilus* showed a better ability to retain the features. After the first month, 40% of the features were retained and another 10% of the hybrids were reverted in the next 4 weeks.

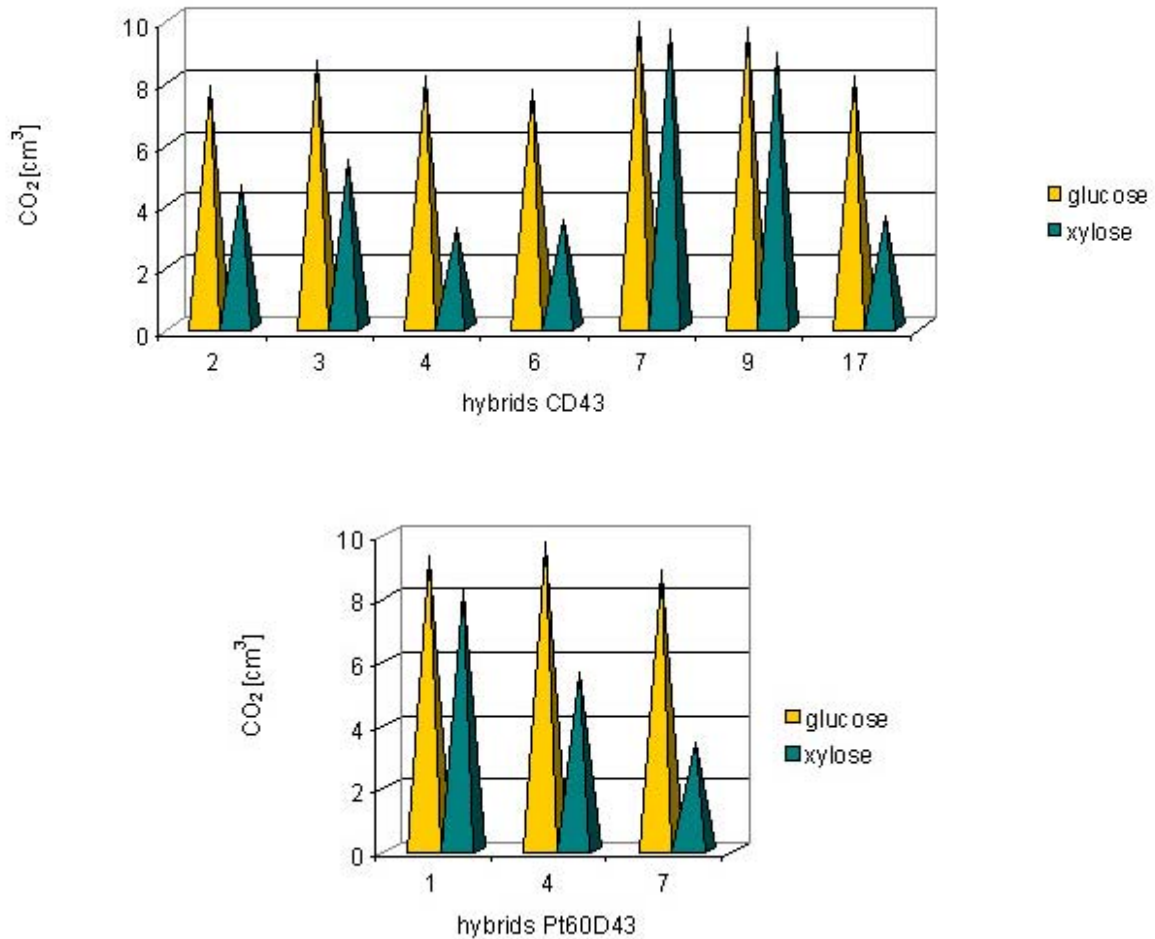
Fig. 2. Stability of the hybrids CD43, Pt32D43 and Pt60D43 during storing



After 4 weeks of storing, there were 7 stable CD43 hybrids (CD43-2, CD43-3, CD43-6, CD43-7, CD43-9, CD43-17) and 3 Pt60D43 hybrids (Pt60D43-1, Pt60D43-4, Pt60D43-7). All of them were characterised by the ability to grow on selection media also after 24 weeks of storing. No stable hybrids of *Saccharomyces cerevisiae* D43 and *Pachysolen tannophilus* ATCC 32691 were obtained. Such hybrids lost their growing ability after 8 weeks of storing.

In the next stage of selection, the aim of which was to select the hybrids with the best abilities to ferment glucose and xylose, the fermenting ability was assessed during a 7-days' fermentation in tubes (Fig. 3).

Fig. 3. Fermentative ability (as cm³ produced CO₂) of the hybrids CD43 and Pt60D43 during fermentation of glucose and xylose (numbers refer to the numbers of hybrids in the groups)



The CD43-7 and CD43-9 hybrids were characterised by the best abilities to ferment glucose and xylose – during 7 days, over 9.7cm³ of CO₂ was accumulated in samples with glucose and 9.0cm³ CO₂ in samples with xylose. The CD43-4, CD43-6 and CD43-17 hybrids had the worst fermenting abilities.

From among the hybrids of *Saccharomyces cerevisiae* 43 and *Pachysolen tannophilus* ATCC 60392, Pt60D43-1 had the best abilities to ferment glucose and xylose – 9.3cm³ and 8.2 cm³ CO₂ was accumulated in the set conditions, respectively.

Most of the hybrids fermented xylose much worse than glucose. 4 hybrids of *Saccharomyces cerevisiae* D43 and *Candida shehatae* ATCC 58779 (CD43-2, CD43-3, CD43-7, CD43-9) and 2 hybrids of *Saccharomyces cerevisiae* D43 and *Pachysolen tannophilus* ATCC 60392 (Pt60D43-1, Pt60D43-4) were selected for further research.

Analysis of genomic DNA of parental strains and their hybrids

A microsatellite primer (GTG)₅ was used for differentiating the interspecific hybrids of *Saccharomyces cerevisiae* D43 and *Candida shehatae* ATCC 58779 and *Pachysolen tannophilus* ATCC 60392. Microsatellite sequences are a type of recurring nucleotide sequences occurring in the non-coding part of the genome of eucariotic organisms, although it may also sometimes occur in exones. It was noticed that microsatellites are extremely different in the number of repetitions of the basic arrangement among the strains and in differentiation at the intraspecies level. The latter feature and the fact that the changes may be easily analysed using PCR methods make microsatellites perfect genetic markers [18]. Baleiras Couto et al. [2] showed the usefulness of microsatellite primers (GAC)₅ and (GTG)₅ in differentiating strains within *Saccharomyces cerevisiae*.

Electrophoretic analysis of the products of amplification of DNA isolated from *Saccharomyces cerevisiae* D43, *Candida shehatae* ATCC 58779 and their hybrids is presented in Fig. 4. The presence of fragments of DNA

(650bp and 400bp), common for both parental strains, was observed in hybrids CD43-2, CD43-3, CD43-7 and CD43-9 (Table 6). Moreover, the hybrids CD43-7 and CD43-9 had regions characteristic for *Candida shehatae* ATCC 58779, namely 1050bp, 800bp and 600bp. The results obtained in the study may suggest a similarity between the hybrids CD43-2, CD43-3 and the strain *Saccharomyces cerevisiae* D43 and a similarity between the hybrids CD43-7, CD43-9 and the strain *Candida shehatae* ATCC 58779, but they cannot be considered 100% hybrids.

Fig. 4. Electrophoretic analysis of the products of amplification of DNA isolated from:
1- *Saccharomyces cerevisiae* D43, 2- *Candida shehatae* ATCC 58779, 3- CD43-2, 4- CD43-3, 5- CD43-7, 6- CD43-9 (M-100bp DNA Ladder Plus) obtained using a microsatellite primer (5' - 3') (GTG)₅

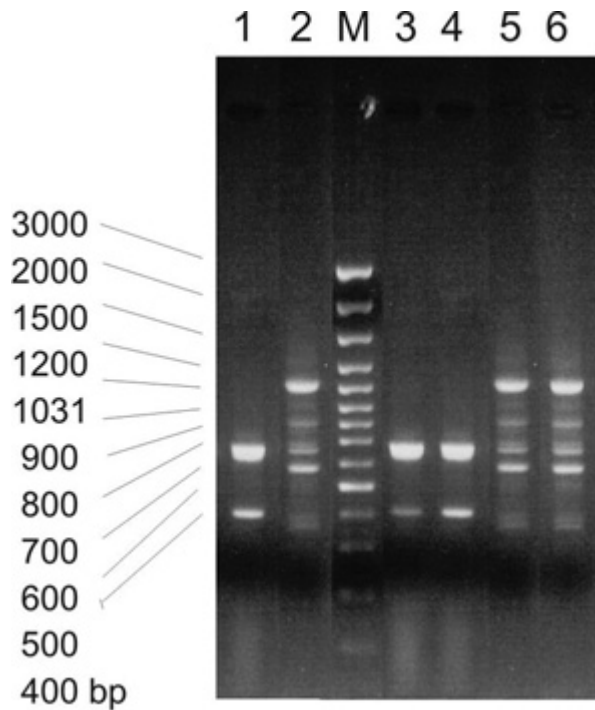


Table 6. The size of DNA fragments of *Saccharomyces cerevisiae* D43, *Candida shehatae* ATCC 58779 and their hybrids obtained in PCR

Yeast	Yeast strain or symbol of hybrid	The size of DNA fragments [bp]				
		1050	800	650	600	400
Parental	<i>Saccharomyces cerevisiae</i> D43			x		x
	<i>Candida shehatae</i> ATCC 58779	x	x	x	x	(x)
Hybrids	CD43-2			x		x
	CD43-3			x		x
	CD43-7	x	x	x	x	(x)
	CD43-9	x	x	x	x	(x)

(x)-poor visible fragment

Electrophoretic analysis of the products of amplification of DNA isolated from *Saccharomyces cerevisiae* D43, *Pachysolen tannophilus* ATCC 60393 and their hybrids is presented in Fig. 5. The hybrids Pt60D43-1 and Pt60D43-4 (Table 7) were characterised by a presence of regions common for both parental forms (650bp and 400bp). Moreover, 1359bp and 600bp fragments, also observed in *Pachysolen tannophilus* ATCC 60392, were identified in the hybrid Pt60D43-1.

Fig.5. Electrophoretic analysis of the products of amplification of DNA isolated from:
1- *Saccharomyces cerevisiae* D43, 2- *Pachysolen tannophilus* ATCC 60392, 3- Pt60D43-1, 4- Pt60D43-4 (M-100bp DNA Ladder Plus) obtained using a microsatellite primer (5' - 3') (GTG)₅

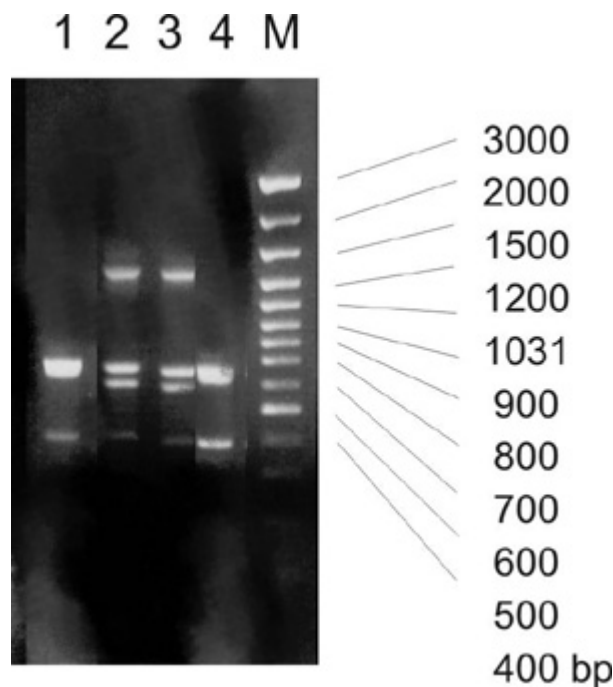


Table 7. The size of DNA fragments of *Saccharomyces cerevisiae* D43, *Pachysolen tannophilus* ATCC 60392 and their hybrids obtained in PCR

Yeast strain	Yeast strain or symbol of hybrid	The size of DNA fragments [bp]			
		1350	650	600	400
Parental	<i>Saccharomyces cerevisiae</i> D43		x		x
	<i>Pachysolen tannophilus</i> ATCC 60392	x	x	x	x
Hybrids	Pt60D43-1	x	x	x	x
	Pt60D43-4		x		x

The use of a primer (GTG)₅ primer did not fully confirm DNA recombination in the strains which were hybridised. The hybrids of *Saccharomyces cerevisiae*, *Candida shehatae* and *Pachysolen tannophilus* were rather selectants with domination of one of the partners in the fusion. The products of fusion may undergo the process of plasmogamy, not karyogamy. It also often happens that single chromosomes of the “weaker” partner are taken over by the dominant strain or that one of the partner strains is polyploidised, which improves its features. Moreover, according to Nečas [15], the reconstruction of cell wall during regeneration of the protoplasts is different than during typical building of a wall during budding, hence different wall structure, chemical composition and also wall features. A rebuild wall of a revertant is not always an exact copy of a primary cell, so the features of the cell may also be different. Protoplasts formation and regeneration of protoplasts may improve the features of microorganisms [4]. Stobińska et al. [23], through regeneration of protoplasts *Schwanniomyces occidentalis* Y671/6, obtained clones whose amolytic activity was even 54% higher than that of the primary strain and whose specific growth rate was 38% higher.

As a result of the presence of DNA regions (650p and 400bp) in both partners in the fusion, it was impossible to determine the origin of the fragments in the hybrids. Most probably, the use of other primers amplifying more selective areas of DNA could bring better results.

CONCLUSIONS

1. Diverse growing abilities of distilling yeasts and xylose-fermenting yeasts in media with xylose as the only carbon source, at 42°C and with 0.01% crystal violet, proved to be useful markers allowing for easy selection of hybrids.
2. Protoplasts formation with Novozym™₂₃₄ and 0.6 M KCl used as an osmotic stabiliser made it possible to obtain over 90% of protoplasts of all lysed yeast strains. The rate of protoplast regeneration was from 48.2% (*Pachysolen tannophilus* ATCC 32691) to 67.3% (*Saccharomyces cerevisiae* D43).
3. 29 hybrids were obtained by protoplast fusion in polyethylene glycol. 6 of these hybrids did not show an ability to convert xylose to ethanol.
4. 4 hybrids of the yeasts *Saccharomyces cerevisiae* D43 and *Candida shehatae* ATCC 58779 (CD43-2, CD43-3, CD43-7, CD43-9) and 2 hybrids of the yeasts *Saccharomyces cerevisiae* D43 and *Pachysolen tannophilus* ATCC 60392 (Pt60D43-1, Pt60D43-4) were selected for genomic DNA analysis after their stability and ability to ferment glucose and xylose were assessed.
5. Although the somatic hybrids CD43-7, CD43-9 and Pt60D43-1 which were obtained effectively fermented both glucose and xylose, the electrophoretic analysis of the products of PCR obtained using a microsatellite primer (GTG)₅ did not confirm DNA recombination of parental strains.

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