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SELECTED BIOTECHNOLOGICAL FEATURES OF HYBRIDS OF *SACCHAROMYCES CEREVISIAE* AND *YAMADAZYMA STIPITIS*

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ABSTRACT

The aim of the research was to investigate the possibility of the use of interspecific hybrids of yeast in ethanol fermentation of media containing xylose. Distilling yeast *Saccharomyces cerevisiae* D43, xylose fermenting yeast *Yamadazyma stipitis* ATCC 58376 and their hybrids: YD43-4, YD43-6, YD43-11 and YD43-12 were used as biological material. The parental strain *Yamadazyma stipitis* ATCC 58376 produced 0.389 g ethanol from 1 g xylose. The hybrids YD43-6 and YD43-11 did not statistically differ from *Yamadazyma stipitis* in respect to efficiency of ethanol from xylose, but produced less xylitol. In the model medium, with the ratio of glucose to xylose (7:3) corresponding to lignocellulitic hydrolysates, the hybrid YD43-6 produced the most ethanol (0.377 g/g).

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

INTRODUCTION

In recent years, the interest in alternative sources of energy, including ethanol, has been growing. To make the price of ethanol competitive as compared to the price of fossil fuels, it is necessary to apply modern technological solutions and cheaper components in its production [5, 29]. The use of lignocellulitic materials,

including plant biomass, agricultural, industrial and municipal wastes or forestry residues for commercial production of ethanol, is especially interesting [16, 35, 37, 46]. The use of microorganisms which effectively utilize all carbohydrates present in lignocellulitic hydrolysates is a major problem. The biggest problems are observed in economic conversion of pentoses, esp. xylose, present in large amounts. *Saccharomyces cerevisiae*, yeast commonly used in distilling industry, do not ferment xylose. Such yeasts as *Pichia (Yamadazyma) stipitis*, *Candida shehatae* and *Pachysolen tannophilus* are characterised by the best ability to produce ethanol from D-xylose [8, 13, 33]. However, their use for fermentation of complex lignocellulitic hydrolysates is not very economic, especially due to a long time of fermentation, low ethanol yield, difficult optimization of physico-chemical parameters of the process and weaker tolerance for numerous inhibitors present in the media [28].

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 [27, 43].

The objective of the present study was to use protoplast fusion to obtain new strains of yeasts characterized by high fermentative ability, good tolerance of ethanol and unfavourable media factors and ability to ferment D-xylose. As a result of protoplast fusion in polyethylene glycol, 13 hybrids of *Saccharomyces cerevisiae* D43 and *Yamadazyma stipitis* ATCC 58376 were obtained, one of them – although characterised by the ability to grow on xylose - did not ferment xylose. Moreover, three other hybrids lost the features gained from the partners in fusion during storage. The remaining hybrids were tested, their fermentative ability in media with glucose and xylose was assessed and the recombination of genetic material was confirmed through the analysis of homology of DNA of parental strains and the hybrids in polymerase chain reaction using microsatellite primer $(GTG)_5$. In case of two hybrids: YD43-6 and YD43-11, genes *XYL*1 and *XYL*2, present also in *Yamadazyma stipitis* ATCC 58376 – a parent fermenting xylose – were observed [2]. Xylose reductase and xylitol dehydrogenase, enzymes coded by genes *XYL*1 and *XYL*2 and also bacterial xylose isomeraze, coded by gene *XYL*A [25, 31], are indispensable in the process of D-xylose fermentation.

In the present study, potential technological applications of the obtained hybrids were tested. For the assessment of selected biotechnological features, out of 7 stable, xylose fermenting hybrids, the hybrids with confirmed presence of genes *XYL*1 and *XYL*2 – YD43-6 and YD43-11 and hybrids YD43-4 and YD43-12, characterised in selection tests by fermentative activity similar to that of hybrids YD43-6 and YD42-11 were chosen.

THE AIM OF THE RESEARCH

The aim of the research was to assess the possibility of the use of interspecific hybrids of distilling and xylose fermenting yeasts in ethanol fermentation of substrates containing xylose.

Selected biotechnological features (growth rate, dynamics of fermenting glucose and xylose, yield of ethanol from glucose and xylose, yield of xylitol from xylose, physiological condition of yeast cells after the process of fermentation) of parental stains and hybrids were assessed in the study.

MATERIALS AND METHODS

Biological material

Distilling yeast *Saccharomyces cerevisiae* D43 from the Collection of Cultures of the Department of Food Storage and Technology (Agricultural University of Wroclaw), xylose fermenting yeasts *Yamadazyma stipitis* ATCC 58376 and their hybrids: YD43-4, YD43-6, YD43-11 and YD43-12, obtained through protoplast fusion in polyethylene glycol [2], were used in the present study.

Growth dynamics

For each culture of strains a curve of the relation of absorbance of the cell suspension to the concentration of dry mass A=f (dry mass of yeast) was determined. Dry mass of yeast was marked using dryer-weight method. For dilutions of suspension of cells from 48 h culture in YM medium, absorbance (λ=560 nm) was measured using a BECKMAN DU-650 spectrophotometer. 10 cm^3 of suspension of cells with known absorbance were transferred to tared weighing bottles with sea sand and dried at 105°C until dry mass was obtained. In the next stage, absorbance (λ=560 nm) was measured from the 3^{rd} to the 30^{th} hour of culture in YM medium. Based on the standard curves A=f (dry mass of yeast), growth curves were determined as a relation of the concentration of biomass to the duration of culture: dry mass of yeast = f (t). The phase of logarithmic growth was determined based on the growth curves. The growth rate μ was calculated using the following formula:

$$\mu = \frac{\ln x_t - \ln x_0}{t_2 - t_1} \quad ,$$

where x_1 - density of cells [dry mass g/dm^3] at time t_2, x_0 - density of cells [dry mass g/dm^3] at time t_1 , t_1 and t_2 – beginning and end of the phase of logarithmic growth [38], respectively.

Dynamics and results of ethanol fermentation

The dynamics and results of ethanol fermentation were assessed at 30°C in media with glucose (100 g/dm^3), xylose (100 g/dm^3) and a mixture of both carbohydrates (70 g/dm^3 glucose and 30 g/dm^3 xylose) enriched with mineral salts (0.5 g/dm^3 $MgSO_4 \cdot 7H_2O$, 2 g/dm^3 $(NH_4)_2SO_4$ and 5 g/dm^3 KH_2PO_4) and yeast extract (5 g/dm^3), inoculated with yeast at the amount of 2 g dry mass yeast/dm^3. Standardisation of inoculum was performed based on standard curves A=f (dry mass yeast). During fermentation, the media with xylose and mixture of glucose and xylose were shaked (150 rpm).

The mass of CO_2 [g] produced after 20, 72 and 168 hours of fermentation in % of total CO_2 [g] produced during fermentation was used as a criterion of the assessment of the dynamics of the process.

After the fermentation, the samples were distilled. The content of ethanol [g/dm^3] in the distillate was marked picnometrically and the yield of ethanol $Y_{et/g}$, $Y_{et/k}$ or $Y_{et/g+k}$ was calculated as the ratio of ethanol content [g] to the initial content of, respectively, glucose, xylose or the mixture of glucose and xylose [g] in the fermentation samples.

Glucose content [g/dm^3] in the samples before fermentation and in the decoctions after distilling was marked using the Nizovkin and Yemielianova method in Soczynski modification [36]. The degree of glucose consumption ΔG [%] was calculated.

Xylose content [g/dm^3] in the samples before fermentation and in the decoctions was marked spectrophotometrically as described by Hashimoto et al. [10]. Absorbance at light wavelength λ=670 nm was measured using a BECKMAN DU-650 spectrophotometer. The degree of xylose consumption ΔK [%] was calculated.

After fermentation, the content of xylitol in the samples with xylose and in those with glucose and xylose was marked. Enzymatic method with BOEHRINGER MANNHEIM (No. 670057) tests was used for the analysis. Absorbance at light wavelength λ=492 nm was measured using a BECKMAN DU-650 spectrophotometer. Xylitol yield $Y_{ksylit/k}$ as a ratio of xylitol content [g] to initial content of xylose [g] in fermentation sample was calculated.

The physiological condition of yeast cells [% inactive and budding cells in relation to total of yeast cells] was assessed using a survival preparation coloured with methylene blue (inactive cells take blue colour) from post-fermentation medium. Yeast cells were calculated in a haemocytometric camera (Thoma system) using a Biolar 2308 microscope.

Statistical analysis

In order to define the relevance of differences between the results obtained in the experiments, one-way analysis of variance in a STATOGRAPHICS Plus Version 6.0 was used.

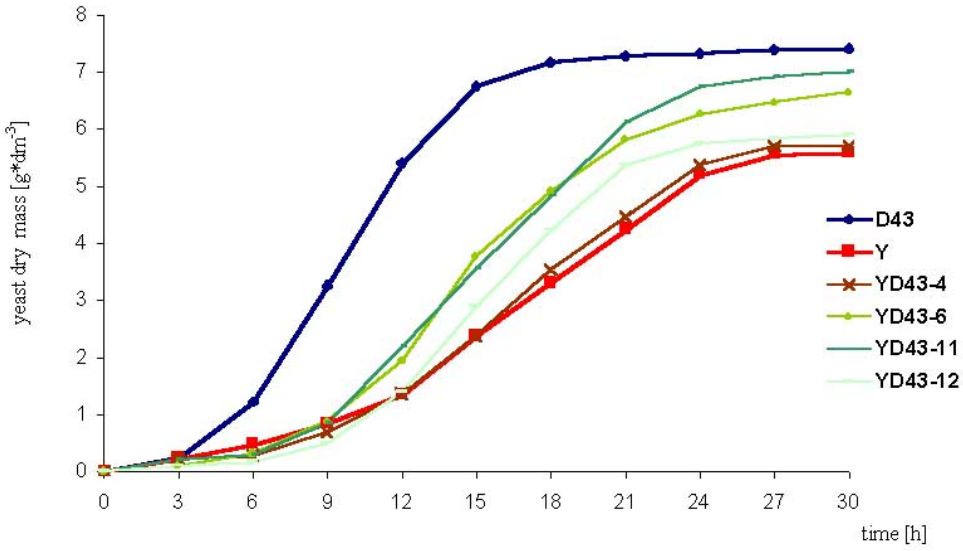
RESULTS

Growth dynamics of parental strains and hybrids

Parental strains *Saccharomyces cerevisiae* D43 and *Yamadazyma stipitis* ATCC 58376 were characterised by different growth dynamics (Fig. 1). The distilling yeasts had a better growth rate than *Yamadazyma stipitis* ATCC 58376. All hybrids used in the experiment were characterised by moderate growth dynamics, better than the strain fermenting xylose, but not as good as *Saccharomyces cerevisiae* D43. Both the parental strain *Yamadazyma stipitis* ATCC 58376 and the hybrids were characterised by longer adaptation phase (9-12 h), whereas in the parental strain *Saccharomyces cerevisiae* D43, the phase of logarithmic growth started in the 6th

hour of cultivation. The xylose fermenting stains reached their phase of stationary growth after about 24-27 h of growth and *Saccharomyces cerevisiae* D43 reached the phase after ca. 18 hours.

Fig. 1. The curves of growth of *Saccharomyces cerevisiae* D43 (D43), *Yamadazyma stipitis* ATCC 58376 (Y) and their hybrids: YD43-4, YD43-6, YD43-11 and YD43-12



During the phase of logarithmic growth, the specific growth rate (Table 1) of parental strains *Saccharomyces cerevisiae* D43, *Yamadazyma stipitis* ATCC 58376 and their hybrids was significantly different from the lowest rate of $0.11h^{-1}$ in *Yamadazyma stipitis* ATCC 58376 to $0.23h^{-1}$ in YD43-6. The growth rate of parental strains was different. The strain *Yamadazyma stipitis* ATCC 58376 grew almost two times slower than *Saccharomyces cerevisiae* D43 ($0.21h^{-1}$). Two of the hybrids discussed in the present study, YD43-6 and YD43-12, inherited from their parental strain *Saccharomyces cerevisiae* D43 the ability to grow fast. The specific growth rate of these hybrids was ca. $0.2h^{-1}$. The specific growth rate of the yeast *Yamadazyma stipitis* ATCC 58376 and the hybrid YD43-4 and YD43-11 was $0.11h^{-1}$.

Table 1. Comparison of the specific growth rate of the yeasts *Saccharomyces cerevisiae* D43, *Yamadazyma stipitis* ATCC 58376 and their hybrids

Yeast	Yeast strain or symbol of hybrid	μ [h^{-1}]
Parental strains	*Saccharomyces cerevisiae* D43	0.21
	Yamadazyma stipitis ATCC 58376	0.11
Hybrids	YD43-4	0.11
	YD43-6	0.23
	YD43-11	0.11
	YD43-12	0.19

Both the parental strain *Yamadazyma stipitis* ATCC 58376 and the hybrids were characterised by longer phase of adaptation to the conditions of media, later moment of reaching the phase of stationary growth and lower biomass yield than *Saccharomyces cerevisiae* D43. Moreover, their growth rate in logarithmic phase, excluding YD43-6 and YD43-12, was lower. The above facts confirm low growth dynamics of strains fermenting xylose.

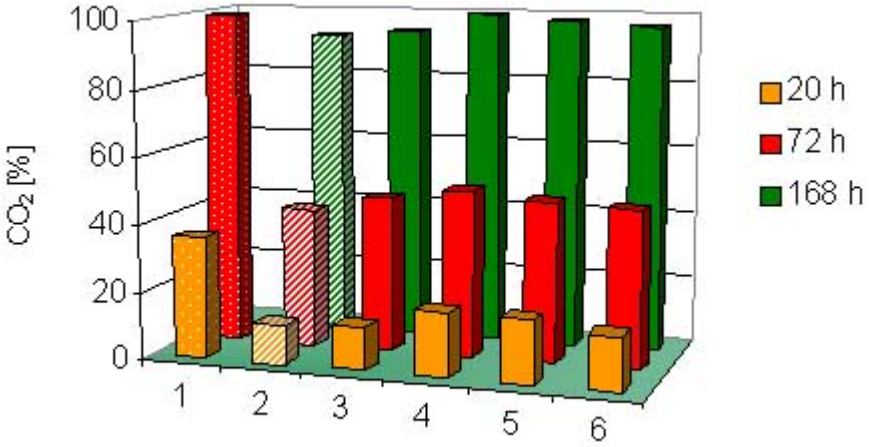
Fermentative ability of strains in media containing various carbon sources

In the next stage, the dynamics of fermentation, ethanol yield, degree of sugar consumption from the medium and physiological condition of yeast cells were assessed. Glucose, xylose and glucose with xylose were used as the only sources of carbon. Moreover, in fermentation of substrates with xylose, the ability of parental strains and hybrids to concentrate in xylitol media was also assessed.

The ability of strains to ferment glucose

The parental strain of the yeast fermenting xylose which was used in the study showed much lower dynamics of fermenting glucose as compared with industrial distilling yeasts (Fig. 2). The yeast *Saccharomyces cerevisiae* D43 fully fermented glucose in 72 hours whereas, after 72 hours, the strain *Yamadazyma stipitis* ATCC 58376 produced only 41.8% of total mass of CO_2. After 168h it produced 92.9%. It should be noted that all the hybrids of *Saccharomyces cerevisiae* D43 and *Yamadazyma stipitis* ATCC 58376 (Fig. 2) were characterised by a higher rate of fermenting glucose than the weaker of the parental strains. The highest rate of fermenting glucose was observed in samples with hybrid YD43-6 in which glucose was fully fermented after 168h.

Fig. 2. Comparison of the dynamics of fermentation of glucose (% CO_2 produced after 20, 72 and 168 h as compared to total CO_2 [g] produced) by:
1 – *Saccharomyces cerevisiae* D43,
2 – *Yamadazyma stipitis* ATCC 58376 and their hybrids:
3 – YD43-4, 4 – YD43-6, 5 – YD43-11, 6 – YD43-12



The strains discussed in the present study significantly differed in the amount of ethanol (presented as practical yield in Table 2) produced by them. The strain *Saccharomyces cerevisiae* D43 was characterised by the highest yield of ethanol from glucose, whereas the strain *Yamadazyma stipitis* ATCC 58376 produced 0.369 g ethanol from 1 g glucose. The yield was significantly statistically lower than in case of distilling yeast – 0.432g/g. The hybrid YD43-6 had the highest yield of ethanol (0.397 g/g), which even exceeded the yield in case of parental strain *Yamadazyma stipitis* ATCC 58376. The hybrid YD42-11 was also characterised by yield of ethanol higher than that of the weaker partner in fusion (0.385 g/g). In the hybrid YD42-12, the feature was not significantly statistically different than in partner *Yamadazyma stipitis* ATCC 58376. The yield of ethanol was slightly different in samples with hybrid YD43-4 (0.350 g/g).

Table 2. The yield of ethanol ($Y_{et/g}$) and the degree of the consumption of glucose by parental strains and hybrids after fermenting a medium containing 100 g/dm^3 glucose

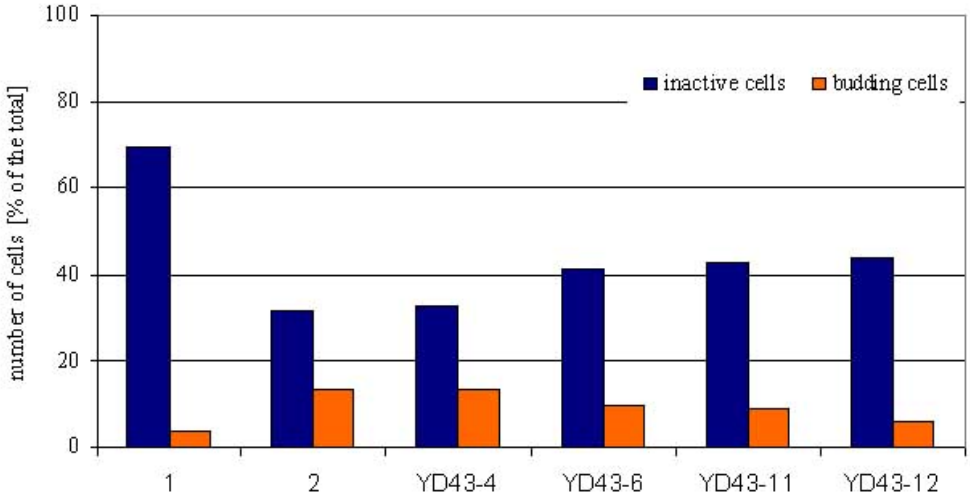
Yeast	Yeast strain or symbol of hybrid	$Y_{et/g}$ [g/g]	Δ G [%]
Parental strains	*Saccharomyces cerevisiae* D43	0.432$_a$	93.2
	Yamadazyma stipitis ATCC 58376	0.369$_d$	81.3
Hybrids	YD43-4	0.350$_e$	74.3
	YD43-6	0.397$_b$	85.4
	YD43-11	0.385$_c$	83.4
	YD43-12	0.368$_d$	80.8

a ÷ e – homogenous groups, NIR=0.0078

The highest consumption of glucose in the process of fermentation was observed in case of *Saccharomyces cerevisiae* D43 (93.2%) and in samples with hybrids YD43-6 and YD43-11. The yeast *Yamadazyma stipitis* ATCC 58376 consumed 81.3% of glucose and the hybrid YD43-12, producing the same amount of ethanol, consumed only 80.8% of glucose.

The physiological condition of yeast cells after the end of ethanol fermentation was different (Fig. 3). The biggest number of inactive cells was observed in samples with *Saccharomyces cerevisiae* D43 (69.3%). In the samples with hybrid YD43-4, the number of cells coloured by methylene blue was the lowest when compared with samples containing the other hybrids.

Fig. 3. Comparison of the physiological condition of the cells of the parental strains and hybrids after the process of ethanol fermentation of glucose (1 - *Saccharomyces cerevisiae* D43, 2 - *Yamadazyma stipitis* ATCC 58376 and their hybrids)



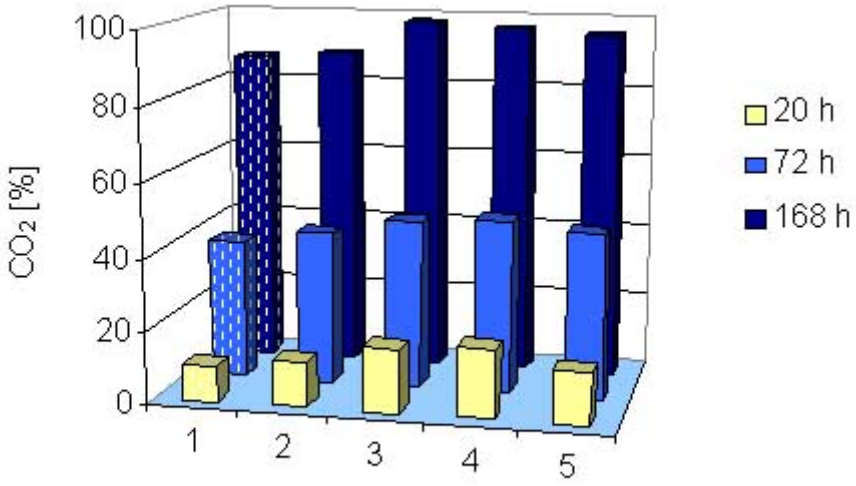
The highest percentage of budding cells after the end of glucose fermentation was observed in samples with strain *Yamadazyma stipitis* ATCC 58376 (13.6%), the lowest percentage was observed in samples with *Saccharomyces cerevisiae* D43 (3.8%). The hybrid YD43-4 was characterised by the biggest percentage of budding cells after the end of the process (13.3%).

It may be concluded that traditional distilling yeast *Saccharomyces cerevisiae* D43 produced ethanol from glucose most effectively. The yield was 0.432 g/g after 72 hours. *Yamadazyma stipitis* ATCC 58376, the strain fermenting xylose, produced 0.369 g/g ethanol during 168 hours. The hybrids YD43-6 and YD43-11 synthesised ethanol more efficiently than the weaker parent, *Yamadazyma stipitis* ATCC 58376.

The ability of strains to ferment xylose

When comparing the hybrids from YD43 group (Fig. 4), it was observed that all of them fermented xylose more dynamically than the parental strain *Yamadazyma stipitis* ATCC 58376. The most outstanding hybrids were YD43-6 and YD43-11. In the 168th hour of the process the amount of CO_2 produced by them was ca. 98%.

Fig. 4. Comparison of the dynamics of fermentation of xylose (% CO_2 produced after 20, 72 and 168h as compared to total CO_2 [g] produced) by:
1 – *Yamadazyma stipitis* ATCC 58376 and their hybrids:
2 – YD43-4, 3 – YD43-6, 4 – YD43-11, 5 – YD43-12



The best yield of ethanol from xylose (Table 3.) was obtained from the strain *Yamadazyma stipitis* ATCC 58376 (0.389 g/g). In case of strains YD43-6 and YD43-11, the differences were not statistically significant. The two remaining hybrids, YD43-12 and YD43-4, produced significantly less ethanol from xylose, 0.355 and 0.314 g/g xylose, respectively.

The degree of xylose consumption (ΔK) is presented in Table 3. The highest consumption of xylose was observed during fermentation with the best ethanol producers – hybrids YD43-11 and YD43-6 (86.7% and 85.3%, respectively) and the strain *Yamadazyma stipitis* ATCC 58376 (84.4%).

Table 3. The yield of ethanol ($Y_{et/g}$), the yield of xylitol ($Y_{ksylit/k}$) and the degree of the consumption of xylose (ΔK) by parental strains and hybrids after fermenting a medium containing 100 g/dm^3 xylose

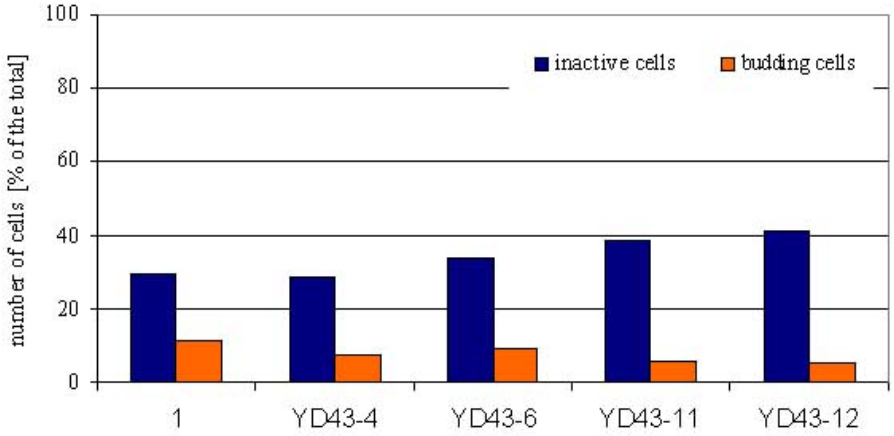
Yeast	Yeast strain or symbol of hybrid	$Y_{et/k}$ [g /g]	Δ K [%]	$Y_{kslit/k}$ [g/g]
Parental strains	*Saccharomyces cerevisiae* D43	-	-	-
	Yamadazyma stipitis ATCC 58376	0.389 $_a$	84.8	0.037 $_A$
Hybrids	YD43-4	0.314 $_c$	69.7	0.012 $_D$
	YD43-6	0.390 $_a$	85.3	0.022 $_{B,C}$
	YD43-11	0.396 $_a$	86.7	0.023 $_B$
	YD43-12	0.355 $_b$	80.1	0.039 $_A$

a ÷ c -homogenous groups, NIR=0.0071,
A ÷ D - homogenous groups, NIR=0.003

The strains used in the study significantly differed in the amount of xylitol, a by-product produced from xylose. The highest amounts were observed in the samples fermented by the parental strain *Yamadazyma stipitis* ATCC 58376 (0.037 g/g). The hybrids differed significantly in their ability to produce this by-product, it was not, however, related to confirmed presence of gene *XYL*1 in their genome, as the strains YD43-6 and YD43-11 with this gene produced significantly less xylitol, both when compared with initial strains and with YD43-12, a hybrid from the same group, which produced the biggest amount of xylitol (0.039 g/g). The fact that the hybrids YD32-4, YD43-6 and YD43-11 produced significantly less xylitol (0.012 g/g, 0.022 g/g and 0.023 g/g, respectively) than the parental strain *Yamadazyma stipitis* ATCC 58376 is worth noting. It indicates a significant improvement of the features of the parental strain, especially as the hybrids YD43-6 and YD43-11 were characterised by the highest yield of ethanol from xylose.

The physiological condition of yeast cells after the end of xylose fermentation is presented in Fig. 5. The lowest number of inactive cells was observed in hybrid YD43-4 and strain *Yamadazyma stipitis* ATCC 58376 (below 30%). The highest number of budding cells was observed in *Yamadazyma stipitis* ATCC 58376 (11.2%).

Fig. 5. Comparison of the physiological condition of the cells of the parental strains and hybrids after the process of ethanol fermentation of xylose (1 - *Yamadazyma stipitis* ATCC 58376 and the hybrids)

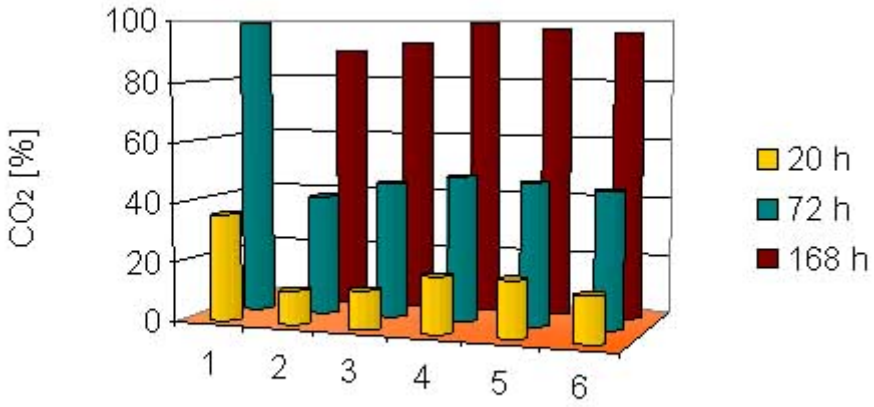


Fermentative ability of strains in the presence of glucose and xylose

The key issue in the assessment of usefulness of the recombined strains to consume xylose in a medium where glucose dominates, as it is the case in processes of fermentation of hydrolysed lignocellulitic materials. The parental strains and hybrids were tested in model media characterised by an arrangement of carbohydrates similar to that in lignocellulitic hydrolysates where the ratio of glucose to xylose was 7:3.

In the model medium with a mixture of sugars, the yeast *Saccharomyces cerevisiae* D43 was characterised by the best fermentation dynamics and the process was completed after 72 hours. The other parental strain was less dynamic and, after 168 h, it produced only 89.7% of total CO_2 mass. The hybrids YD43-4, YD43-6, YD43-11 and YD43-12 were characterised by better fermentation dynamics than *Yamadazyma stipitis* ATCC 58376, the weaker partner in the fusion. The hybrids YD43-6 and YD43-11 fermented the mixture of sugars most effectively and the process was completed after 168 hours (Fig. 6).

Fig. 6. Comparison of the dynamics of fermentation of glucose and xylose in mixed medium (% CO_2 produced after 20. 72 and 168 h as compared to total CO_2 [g] produced) by: 1 – *Saccharomyces cerevisiae* D43, 2 – *Yamadazyma stipitis* ATCC 58376 and their hybrids: 3 – YD43-4, 4 – YD43-6, 5 – YD43-11, 6 – YD43-12.



The dynamics of fermentation was reflected in the yield of ethanol (Table 4). The biggest yield of ethanol from the mixture of glucose and xylose was observed in the hybrid YD43-6 – 0.377 g/g. The other hybrids produced ethanol less effectively than *Yamadazyma stipitis* ATCC 58376 (0.372 g/g). It should be noted that the hybrids YD43, able to ferment xylose, were characterised by a better yield of ethanol than *Saccharomyces cerevisiae* D43.

Consumption of glucose (Table 4) in a medium with a mixture of sugars was very good in case of all strains, including those able to ferment xylose (from 98.8% in case of the strain YD43-6 to 97.2% in case of parental strain *Yamadazyma stipitis*). *Saccharomyces cerevisiae* D43 consumed glucose in 94.9% (as compared to 93.2% during fermentation of glucose as the sole carbon source). The degree of xylose consumption was, however, low – from 25.4% in case of YD43-4 to 41.8% in case of YD43-6.

Table 4. The yield of ethanol ($Y_{et/g+k}$), the yield of xylitol ($Y_{ksylit/k}$) and the degree of the consumption of glucose (ΔG) and xylose (ΔK) by parental strains and hybrids after fermenting a medium containing 70 g/dm^3 glucose and 30 g/dm^3 xylose

Yeast	Yeast strain or symbol of hybrid	$Y_{et/g+k}$ [g /g]	Δ G [%]	Δ K [%]	$Y_{kslit/k}$ [g/g]
Parental strains	*Saccharomyces cerevisiae* D43	0.313 $_e$	94.9	-	-
	Yamadazyma stipitis ATCC 58376	0.372 $_b$	97.2	40.6	0.078 $_A$
Hybrids	YD43-4	0.348 $_d$	97.8	25.4	0.025 $_D$
	YD43-6	0.377 $_a$	98.8	41.8	0.038 $_C$
	YD43-11	0.366 $_c$	98.3	35.8	0.046 $_B$
	YD43-12	0.348 $_d$	97.5	30.1	0.076 $_A$

a - e - homogenous groups, NIR=0.0047, A - D - homogenous groups, NIR=0.0035

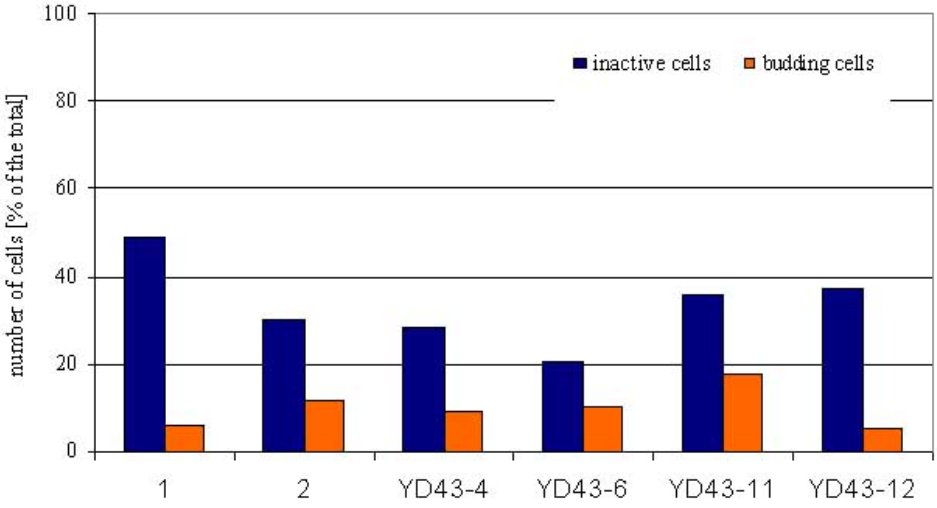
The strains used in the present study produced more xylitol in the medium. The yield of this by-product was from 0.025 g/g in case of the hybrid YD43-4 to 0.078 g/g in case of the parental strain. Similarly, as in the process of fermentation of xylose, for similar yield of ethanol and the degree of xylose consumption, the yield of xylitol was significantly different. It should be noted that the amount of xylitol produced in the medium with glucose and xylose (7:3) was two times higher as compared to the medium with xylose only and the degree of xylose consumption was 44 – 51 % lower (Table 5).

Table 5. Comparison of the yield of xylitol ($Y_{ksylit/k}$) and the degree of the consumption of xylose (ΔK) after fermenting xylose and a mixture of glucose and xylose

Yeast strain or symbol of hybrid	XYLOSE		GLUCOSE + XYLOSE	
	$Y_{ksylit/k}$ [g/g]	Δ K [%]	$Y_{ksylit/k}$ [g/g]	Δ K [%]
Saccharomyces cerevisiae D43	-	-	-	-
Yamadazyma stipitis ATCC 58376	0.037	84.8	0.078	40.6
YD43-4	0.012	69.7	0.025	25.4
YD43-6	0.022	85.3	0.038	41.8
YD43-11	0.023	86.7	0.046	35.8
YD43-12	0.039	80.1	0.076	30.1

Hybrids YD43-6, YD43-4 and the parental strain *Yamadazyma stipitis* ATCC 58376 were characterised by the best physiological condition after fermenting the mixture of glucose and xylose. The biggest amount of inactive cells (48.9%) was observed in *Saccharomyces cerevisiae* D43 (Fig. 7).

Fig. 7. Comparison of the physiological condition of the cells of the parental strains and hybrids after the process of ethanol fermentation of the mixture of glucose and xylose (1 – *Saccharomyces cerevisiae* D43, 2 - *Yamadazyma stipitis* ATCC 58376 and their hybrids)



The highest yield of ethanol from the mixture of glucose and xylose was obtained using hybrid YD43-6 – 0.377 g/g, which also produced xylitol (0.038 g/g), and was much less than in case of the parental strain *Yamadazyma stipitis* ATCC 58376 (0.078 g/g). The other hybrids produced ethanol less effectively than *Yamadazyma stipitis* ATCC 58376 and, with the exception of the hybrid YD43-12, produced less amounts of the by-product.

Based on the presented results, the hybrid YD43-6 was selected as a strain with potential possibility to be used for conversion of xylose to ethanol.

DISCUSSION

In recent years there have been numerous attempts, using protoplast fusion and such novel recombination techniques as gene cloning, at constructing strains that effectively ferment D-xylose. After protoplast fusion Heluane et al. [11] obtained hybrids of *Saccharomyces cerevisiae* and *Pachysolen tannophilus* which morphologically resembled *Saccharomyces cerevisiae* and, as both parental strains, had the ability to assimilate sugars. These hybrids, however, did not assimilate D-xylose. Johannsen et al. [14] fused cells of the same strain and obtained polyploids *Candida shehatae* CBS 2779 which produced ethanol from D-xylose slightly faster. The fusion of protoplasts of the strain *Saccharomyces cerevisiae* with mutants *Candida shehatae* and *Pichia stipitis* induced by Gupthar [7] resulted in creation of mononucleic fusants which, however, were not stable.

In 1991 the gene coding reductase of *XYL*1 xylose from the yeast *Pichia stipitis* was cloned for the first time. Its fusion with *Saccharomyces cerevisiae* did not, however, bring the expected results – the yeast *Saccharomyces* did not ferment xylose to ethanol. Higher activity of reductase or expression of *XYL*1 gene in the recombinants *Saccharomyces cerevisiae* resulted in better yield of xylitol [1, 4, 9, 17, 22, 39]. It should be noted that xylitol, due to its physical and chemical qualities, is frequently used as a sweetener in production of diet food, spreads and pills [32]. Nevertheless, biosynthesis of xylitol during alcohol fermentation of xylose reduces the yield of ethanol.

Gene *XYL*2, coding the NAD-dependant xylitol dehydrogenase in *Pichia stipitis*, was also transformed to the genome of *Saccharomyces cerevisiae* [40, 47] and Walfridsson et al. [41] introduced to *Saccharomyces cerevisiae* a bacteria gene *XYL*A from *Thermus thermophilus*, coding isomerase of xylose. Fermentation was conducted at 85°C and the main products were xylitol and acetic acid.

To obtain transformants effectively producing ethanol from xylose, not only genes *XYL*1 and *XYL*2 were cloned, but also sequences coding kinase of xylulose – an enzyme catalysing phosphorilation of xylose to xylulose-5-phosphoran [12, 18, 26] and also genes coding the enzymes of the pentose phosphate pathway and glycolysis [15, 20, 21, 24], e.g. transketolase (*TKL*1) [23] and transaldolase (*TAL*1) [42], dihydroorotate dehydrogenase (*URA*1) [34], orotidine-5'-phosphate decarboxylase (*URA*3) [45] or alcoholic dehydrogenase [3, 30]. Although

sequencing and cloning a gene is, nowadays, not very difficult, selecting a proper vector for transformation and invoking a desirable expression of gene in the host cell is still problematic.

The interspecific hybrids of *Saccharomyces cerevisiae* D43 and *Yamadazyma stipitis* ATCC 58376: YD43-6 and YD43-11 with a confirmed presence of genes coding xylose reductase and xylitol dehydrogenase presented in the present study deserve, in the context of scientific publications, special attention. They produced ethanol from xylose giving a yield corresponding to that of the parental strain *Yamadazyma stipitis* ATCC 58376 (0.389 g/g) and the yield of xylitol was over 40% lower than in case of the parent. Improved dynamics of xylose fermentation resulting from fusion of protoplasts and the strain of distilling yeast, making it possible to shorten the fermentation period from 10 to 7 days, was observed. In model media with a mixture of glucose and xylose (7:3), the hybrid YD43-6 was characterised by the best features and during 7 days its ethanol yield was 0.377 g/g and xylitol yield was 0.038 g/g.

Further research should be devoted to improvement of the process of fermentation of lignocellulitic hydrolysates whose complex composition may have various influences on the fermentative activity and physiological condition of the hybrids. In sugar mixtures, with a composition corresponding to that of lignocellulitic hydrolysates, there takes place sequence assimilation of sugars in which hexoses are preferentially fermented before D-xylose [44]. Such model of fermentation results mainly from glucose catabolite repression [6], hence xylose is consumed only when there is little glucose in the medium. Lowered degree of consumption of xylose from a model fermentative medium containing glycosis and xylose (7:3) as compared to the consumption of xylose during fermentation in a medium containing only xylose was observed in the present study as well.

Although numerous research teams have been recently working on effective bioconversion of lignocellulitic materials to ethanol, it still remains a very important task for biotechnology. Positive results will be valuable not only for economy but for ecology as well.

CONCLUSIONS

1. The strains of xylose fermenting yeasts, both *Yamadazyma stipitis* ATCC 58376 and their hybrids, were characterised by lower growth dynamics and slower cultivation of biomass as compared to the strain *Saccharomyces cerevisiae* D43.
2. In media with glucose as the sole carbon source, the strains of yeasts able to ferment xylose – parental strain and hybrids – were characterised by lower dynamics of fermentation and lower yield of ethanol as compared to *Saccharomyces cerevisiae* D43. The physiological condition of cells of those strains after the end of the process was, however, better than that of the cells of *Saccharomyces cerevisiae* D43.
3. From among the hybrids used in the present study, the hybrids YD43-6 and YD43-11 (with a confirmed presence of genes *XYL*1 and *XYL*2) fermented glucose and xylose most dynamically, were characterised by the highest yield of both glucose and xylose. Their yield of ethanol from xylose did not statistically differ from that obtained by *Yamadazyma stipitis* but the yield of xylitol was lower.
4. The hybrid YD43-6, when compared to the parental strain *Yamadazyma stipitis* ATCC 58376 and other hybrids from group YD43, produced ethanol most effectively in the medium with a ratio of glucose to xylose corresponding to that of lignocellulitic hydrolysates (7:3). When compared to *Yamadazyma stipitis* ATCC 58376, it also produced less xylitol, but after fermentation its cells were characterised by a worse physiological condition than the cells of the parental strain.

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