Electronic Journal of Polish Agricultural Universities is the very first Polish scientific journal published exclusively on the Internet, founded on January 1, 1998 by the following agricultural universities and higher schools of agriculture: University of Technology and Agriculture of Bydgoszcz, Agricultural University of Cracow, Agricultural University of Lublin, Agricultural University of Poznan, Higher School of Agriculture and Teacher Training Siedlce, Agricultural University of Szczecin, and Agricultural University of Wroclaw.

ELECTRONIC JOURNAL OF POLISH AGRICULTURAL UNIVERSITIES

2003
Volume 6
Issue 2
Series BIOTECHNOLOGY

Copyright © Wydawnictwo Akademii Rolniczej we Wroclawiu, ISSN 1505-0297
CHMIELEWSKA J., DZIUBA E. 2003. RESPIRATORY DEFICIENT MUTANTS OF XYLOSE-FERMENTING YEAST – OBTAINMENT AND FEATURES **Electronic Journal of Polish Agricultural Universities**, Biotechnology, Volume 6, Issue 2.
Available Online http://www.ejpau.media.pl

RESPIRATORY DEFICIENT MUTANTS OF XYLOSE-FERMENTING YEAST – OBTAINMENT AND FEATURES

Joanna Chmielewska, Ewelina Dziuba
Department of Food Storage and Technology, Agricultural University of Wrocław, Poland

ABSTRACT
INTRODUCTION
MATERIALS AND METHODS
RESULTS AND DISCUSSION
CONCLUSIONS
REFERENCES

ABSTRACT

The aim of the present research work was to obtain RD mutants of yeast *Pichia stipitis*, *Yamadazyma stipitis*, *Candida shehatae* and *Pachysolen tannophilus* and to estimate fermentative activity of mutants. Ethidium bromide and acriflavine were used as mutagens.

119 RD mutants were isolated. After storage 11 mutants of *Yamadazyma stipitis* ATCC 58376, 7 mutants of *Pichia stipitis* 1 and 10 mutants of *Pichia stipitis* 2 showed the feature *petite*. Neither using ethidium bromide nor acriflavine stable RD mutants of *Candida shehatae* and *Pachysolen tannophilus* strains were obtained.

Mutants of *Yamadazyma stipitis* produced ethanol from glucose with the yield similar to parental strain (87.1÷99.4% parental strain yield), but xylose - with 16.4÷46.6% lower yield. Mutants of *Pichia stipitis* 1 and *Pichia stipitis* 2 were characterized by weak ethanol yield from glucose (respectively 22.0÷58.8% and 20.0÷58.8% less than parental strains) and xylose (respectively 56.7÷76.8% and 40.3÷66.9% less).

Key words: distilling, xylose-fermenting yeast, respiratory deficient mutants

INTRODUCTION

Ethanol has been known for a long time, being perhaps one of the oldest products obtained through traditional biotechnology. Increasing interest in using ethanol as attractive, sustainable energy source mobilises searching for a cheaper substrate, such as lignocellulose biomass: agricultural waste (sugar cane bagasse, wheat straw, corn stalks, soybean residues), industrial waste (pulp and paper industry), forestry residues, municipal solid waste etc., which could make ethanol production more competitive with fossil fuel. One important requirement is an efficient microorganism able to ferment a variety of sugars – pentoses and hexoses- as well as tolerate stress conditions [4, 11, 17]. Much progress has been made since the discovery that same yeasts can ferment D-xylose to ethanol. Three of yeast species: *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* have been studied extensively, but they are sensitive to ethanol and inhibitors present in hydrolysates of lignocellulose. *Saccharomyces cerevisiae*, a yeast traditionally used in the fermentation industry can not ferment xylose [8].

In order to improve fermentative ability of yeast strains genetic method could be used [5, 6]. One of the easiest and cheapest is the protoplast fusion technique, which is especially useful when target characteristics for improvement of strain are determined by several genes and their genetic backgrounds have not been well studied, as is usual with industrial yeast. Despite the requirement of genetic markers in parental strains and the difficulty in introducing certain desired genes, protoplast fusion will be the most practical and convenient technique for yeast improvement if efficient systems for fusants selections are developed [10, 13]. RD mutation could be one of genetic markers used for selection of parental strains and hybrids after protoplast fusion.

The aim of the present research work was to obtain RD mutants of xylose-fermenting yeast and to estimate their fermentative activity.

MATERIALS AND METHODS

Microorganisms and cultivation conditions

Biological material was composed of xylose-fermenting yeast strains: *Pichia stipitis* 1 and 2 - obtained from professor B. Achremowicz and *Yamadazyma stipitis* ATCC 58376, *Candida shehatae* ATCC 58779, *Pachysolen tannophilus* ATCC 32691 and ATCC 60392 from American Type Culture Collection (Rockville, USA). Yeast strains were stored on agar YM slants [1] at 4°C. Microorganisms were precultured for 48h at 30°C in liquid medium YM.

Receiving RD mutants of xylose-fermenting yeast

In order to acquire RD mutants of yeast *Yamadazyma stipitis* ATCC 58376, *Pichia stipitis* 1, *Pichia stipitis* 2, *Candida shehatae* ATCC 58779, *Pachysolen tannophilus* ATCC 32691 and *Pachysolen tannophilus* ATCC 60392 ethidium bromide (10 μg/ml) and acriflavine in concentrations 2, 4 or 6 μg/ml were used. A loopful inoculum from YM slant was transferred to the medium with mutagen.

Media with ethidium bromide (10 g/l yeast extract, 10 g/l peptone, 20 g/l glucose, 10 μg/ml ethidium bromide, pH = 5) were incubated at 30°C in darkness for 48h, then centrifuged, and the obtained biomass was replaced in fresh medium with ethidium bromide. After 24h of incubation at 30°C in darkness the procedure was repeated [9].

Media with acriflavine (20 g/l glucose, 1.8 g/l peptone, 1 g/l KH_2PO_4, 1.5 g/l $(NH_4)_2HPO_4$, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 2 g/l yeast extract and acriflavine in concentrations 2, 4 or 6 μg/ml (pH =5)) were incubated at 30°C in darkness for 48h [12].
After incubation in medium with mutagen, the cultures were diluted and spread on Petri dishes with YPG medium (10 g/l yeast extract, 10 g/l peptone, 20 g/l glucose, 20 g/l agar). After 4 days of incubation at 30°C colonies were replicated on media YPG and YP-GLYCEROL (10 g/l yeast extract, 10 g/l peptone, 20 g/l glycerol, 20 g/l agar) in order to select RD mutants. Colonies unable to grown on medium with glycerol as the sole carbon source were isolated from medium YPG as RD mutants [9, 12].

Frequency of mutation was calculated as % of colonies not able to grow on medium with glycerol in relation to numbers of colonies growing on YPG medium. The obtained RD mutants were stored on medium YM at 4°C. After 2, 4, 8 and 12 weeks of storage, the stability of RD mutation was examined by estimation of the possibility to grow on medium YP-GLYCEROL. The stability of RD features was confirmed by inability of strains to grow on glycerol as the sole carbon source.

Effects of RD mutation on fermentation

Fermentative activity of RD mutants, in comparison with parental strains, was examined in the fermentation tests at 30°C in media with glucose (100 g/l) or xylose (100 g/l), supplemented with $MgSO_4 \cdot 7H_2O$ (0.5 g/l), $(NH_4)_2SO_4$ (2 g/l), KH_2PO_4 (5 g/l) and yeast extract (5 g/l) (pH=5). Media were autoclaved for 20 minutes at 121°C. Xylose solution was prepared separately and added to the fermentation medium. Parental strains and RD mutants were precultured in medium YM. The fermentations were carried out in 300 ml Erlenmeyer flasks, in which 100 ml fermentation medium was inoculated with 5 ml suspension of yeast cells. Xylose fermentations were conducted on a water-bath rotary shaker at 150 rpm. Ethanol evaporation was prevented by rubber stoppers with fermentative tubes filled with 50% H_2SO_4.

In this study the comparison of the ethanol yields produced by parental strains and theirs RD mutants in glucose ($Y_{et/g}$ [g/g]) and xylose ($Y_{et/k}$ [g/g]) medium was made. The physiological condition of yeast cells after glucose and xylose fermentation (% of budding cells and inactive cells (staining with methylene blue) against the total number of cells) was determined as well.

To designate statistically significant differences between the results of experiences one-way analyses of variance with STATGRAPHICS Plus Version 6.0 were performed. Homogenous groups (not statistically significant different) were marked with symbols: A, B, C,…

RESULTS AND DISCUSSION

One of the selective markers, making it possible to select intergeneric, intrageneric as well as interspecific hybrids after protoplasts fusion, can be RD mutation- not full respiratory abilities [14, 15, 16]. RD mutants of *Yamadazyma stipitis* ATCC 58376, *Pichia stipitis* 1, *Pichia stipitis* 2, *Candida shehatae* ATCC 58779, *Pachysolen tannophilus* ATCC 32691 and *Pachysolen tannophilus* ATCC 60392 yeast were obtained by using two mutagens- acriflavine and ethidium bromide. Acriflavine acts on growing cells, while ethidium bromide both on growing and non-growing cells, causing in this way more changes in mitochondrial DNA.

Acriflavine was used in three concentrations and the highest concentration of acriflavine caused mutation with good effect only for *Candida shehatae* ATCC 58779 strain, but did not effect the obtaining of RD mutants *Pichia stipitis* 1, *Pichia stipitis* 2 and *Pachysolen tannophilus* ATCC 60392 (Table 1).

Table 1. Number of RD mutants yeast obtained in ethidium bromide (10 µg/ml) and acriflavine (2 µg/ml, 4 µg/ml, 6 µg/ml) containing medium

Yeast strain	mutagens				
	ethidium bromide	acriflavine			
	10 µ g/ml	2 µ g/ml	4 µ g/ml	6 µ g/ml	Σ
Yamadazyma stipitis ATCC 58376	9	5	8	2	15
Pichia stipitis (1)	4	6	7	-	13
Pichia stipitis (2)	9	10	3	-	13
Candida shehatae ATCC 58779	8	3	5	10	18
Pachysolen tannophilus ATCC 32691	3	-	2	1	3
Pachysolen tannophilus ATCC 60392	7	6	11	-	17

In total, 119 RD mutants were isolated: 24 RD mutants of *Yamadazyma stipitis* ATCC 58376 yeast, 17 RD mutants of *Pichia stipitis* 1, 22 RD mutants of *Pichia stipitis* 2, 26 RD mutants of *Candida shehatae* ATCC 58779, 6 RD mutants of *Pachysolen tannophilus* ATCC 32691 and 24 RD mutants of *Pachysolen tannophilus* ATCC 60392 (Table 1).

The lowest concentration of acriflavine - 2 µg/*ml* - was enough for *Pichia stipitis* 2, but too little to cause respiratory defect in *Pachysolen tannophilus* ATCC 32691. Increasing it to 4 µg/ml improved mutagenic affectivity for *Yamadazyma stipitis* ATCC 58376, *Candida shehatae* ATCC 58779 and *Pachysolen tannophilus* ATCC 60392. The greatest acriflavine concentration -6 µg/ml - was sufficient for induction of mutation in

strains *Candida shehatae* ATCC 58779 and *Pachysolen tannophilus* ATCC 32691, but this mutagen concentration did not effect the obtainment of RD mutants of *Pichia stipitis* 1, *Pichia stipitis* 2 and *Pachysolen tannophilus* ATCC 60392 yeast.

In order that RD mutation could be used to mark one from partners of fusion, this feature has to be stable. For that reason the stability of 119 obtained RD mutants after 2, 4, 8 and 12 weeks of storage was estimated.

All RD mutants of yeast *Candida shehatae* ATCC 58779 lost the feature posted by mutation after 8 weeks of storage, and mutants received with the use of acriflavine in concentration 2 µg/ml lost the *petite* features already after 4 weeks. Also mutants of both strains *Pachysolen tannophilus* proved unstable, independently from the mutagen used. Yeast strain *Pachysolen tannophilus* ATCC 60392, subjected to activity of acriflavine in the highest concentrations used in the experiment, grew neither on medium YPG, nor on medium with glycerol. Strain *Pachysolen tannophilus* ATCC 32691 proved the most sensitive to unprofitable external factors.

From among RD mutants of yeast *Yamadazyma stipitis* ATCC 58376, obtained with ethidium bromide, in examined period of the time, the feature RD kept about 30%. The lowest acriflavine concentration made it possible to keep the stability of all obtained mutants during 12 weeks. Part of mutants *Pichia stipitis* 1 and *Pichia stipitis* 2 yeast received with ethidium bromide and with the lowest acriflavine concentration lost RD feature in the first period of storage, whereas the remaining mutants were stable to the end of the observation. Using middle concentrations of acriflavine at both strains of *Pichia stipitis* allowed to keep about 40% of obtained RD mutants, but the highest concentration of this mutagen acted lethal on the cells of these strains. Yeast strains *Pichia stipitis* 1 and *Pichia stipitis* 2 subjected to the mutation with 6 µg/ml acriflavine did not show abilities to growth neither on medium YPG, nor on YP-GLYCEROL medium.

In conclusion, increased acriflavine concentration in cultivation medium influenced negatively the vitality of yeast *Pichia stipitis* 1, *Pichia stipitis* 2 and *Pachysolen tannophilus* ATCC 60392 and the stability RD of mutation at examined yeast strains, with the exception of yeast *Yamadazyma stipitis* ATCC 58376. Stable respiratory mutants of yeast *Candida shehatae* ATCC 58779, *Pachysolen tannophilus* ATCC 32691 and *Pachysolen tannophilus* ATCC 60392 were not obtained. *Pichia stipitis* and *Yamadazyma stipitis* proved petite positive and RD mutant inducing efficiency of acriflavine is concentration dependent. All the other species examined are petite negative.

After 12 weeks of storage 11 mutants of *Yamadazyma stipitis* ATCC 58376 yeast (in this 3 obtained with ethidium bromide), 7 mutants of *Pichia stipitis* 1 yeast and 10 mutants of *Pichia stipitis* 2 yeast (in this respectively- 2 and 4 obtained with ethidium bromide) showed the *petite* feature.

Estimation of fermentative activity of RD mutants

A very essential feature of RD mutant, as a potential partner of fusion and donor of genetic material, is its ability to effectively produce ethanol from glucose, and especially from xylose and to keep good physiological condition during the process of fermentation. Because of this, all the obtained RD mutants were also subjected to fermentation tests in media containing glucose and xylose.

Ethanol yield after fermentation of glucose and xylose obtained by RD mutants of *Yamadazyma stipitis* ATCC 58376 yeast is shown in table 2. The ethanol yield from glucose for respiratory mutants yeast *Yamadazyma stipitis* ATCC 58376 varied between 0.319 g/g in case of RD_{4A} and 0.364 g/g in case of RD_{6A}. The investigated RD mutants reached 87.1÷99.4 % yield attained in similar conditions by *Yamadazyma stipitis* ATCC 58376 with full respiratory abilities (0.366 g/g).

Table 2. The comparison of ethanol yields from glucose ($Y_{et/g}$) and xylose ($Y_{et/k}$) by *Yamadazyma stipitis* ATCC 58376 yeast strain and its RD mutants

	$Y_{et/g}$ [g/g]	$Y_{et/k}$ [g/g]
Yamadazyma stipitis ATCC 58376	0.366 ^{A}	0.378 ^{A}
RD mutants		
RD_{1b}	0.321 ^{D}	0.307 ^{C}
RD_{2b}	0.355 ^{B}	0.264 ^{F}
RD_{3b}	0.344 ^{C}	0.245 ^{H}
RD_{4a}	0.319 ^{D}	0.302 ^{D}
RD_{5a}	0.354 ^{B}	0.287 ^{E}
RD_{6a}	0.364 ^{A}	0.211 ^{J}
RD_{7a}	0.322 ^{D}	0.202 ^{K}
RD_{8a}	0.359 $^{A.B}$	0.231 ^{I}
RD_{9a}	0.343 ^{C}	0.250 ^{G}
RD_{10a}	0.361 ^{A}	0.316 ^{B}
RD_{11a}	0.363 ^{A}	0.308 ^{C}

*mutagen: a - acriflavine, b - ethidium bromide
A, B, C, …. - homogenous groups

Ethanol yield from xylose in case of the strains mentioned earlier was from 0.202 g/g (for RD_{7A}) to 0.316 g/g (for RD_{10A}), what determined 53.4÷83.6 % yield of ethanol from xylose for yeast *Yamadazyma stipitis* ATCC 58376 (0.378 g/g).

Table 3 presents ethanol yield after fermentation of glucose and xylose by RD mutants yeast *Pichia stipitis* 1. The yield of ethanol from glucose was from 0.073 g/g in case of mutant RD_{3A} to 0.138 g/g in case of RD_{6A}. In comparison with parental strain *Pichia stipitis* 1 (0.177 g/g) RD mutants produced ethanol from glucose with lower yield: 0.039–0.104 g/g.

The yield of ethanol from xylose for this group of strains reached the values from 0.038 g/g (for RD_{1B}) to 0.071 g/g (for RD_{3A}) and only just 23.2÷43.3% yield of strain *Pichia stipitis* 1 (0.164 g/g).

Table 3. The comparison of ethanol yields from glucose ($Y_{et/g}$) and xylose ($Y_{et/k}$) by *Pichia stipitis* 1 yeast strain and its RD mutants

	$Y_{et/g}$ [g/g]	$Y_{et/k}$ [g/g]
Pichia stipitis 1	0.177 ^{A}	0.164 ^{A}
RD mutants		
RD_{1b}	0.122 ^{C}	0.038 ^{E}
RD_{2b}	0.083 ^{E}	0.041 ^{E}
RD_{3a}	0.073 ^{F}	0.071 ^{B}
RD_{4a}	0.118 ^{C}	0.063 ^{C}
RD_{5a}	0.099 ^{D}	0.048 ^{D}
RD_{6a}	0.138 ^{B}	0.052 ^{D}
RD_{7a}	0.080 ^{E}	0.049 ^{D}

*mutagen: a - acriflavine, b - ethidium bromide
A, B, C, …. - homogenous groups

The yield of ethanol from glucose for mutants yeast *Pichia stipitis* 2 (Table 4) reached the value from 0.068 g/g in case of RD_{1B} to 0.132 g/g for the most efficiently fermenting glucose mutant RD_{4B}. In comparison – the yield of parental strain *Pichia stipitis* 2 in similar conditions was about 20÷58.8% greater (0.165 g/g).

The yield of ethanol from xylose in case of described strains was from 0.051 g/g for RD_{6A} to 0.092 g/g for RD_{8A}, which was 33.1÷59.7% of the yield of parental strain *Pichia stipitis* 2 (0.154 g/g) in similar conditions of xylose fermentation.

Table 4. The comparison of ethanol yields from glucose ($Y_{et/g}$) and xylose ($Y_{et/k}$) for *Pichia stipitis* 2 yeast strain and its RD mutants

	$Y_{et/g}$ [g/g]	$Y_{et/k}$ [g/g]
Pichia stipitis 2	0.165 ^{A}	0.154 ^{A}
RD mutants		
RD_{1b}	0.068 ^{G}	0.054 ^{G}
RD_{2b}	0.079 ^{F}	0.070 ^{E}
RD_{3b}	0.082 ^{F}	0.062 ^{F}
RD_{4b}	0.132 ^{B}	0.083 ^{C}
RD_{5a}	0.079 ^{F}	0.065 $^{E.F}$
RD_{6a}	0.119 ^{C}	0.051 ^{G}
RD_{7a}	0.115 ^{C}	0.063 ^{F}
RD_{8a}	0.131 ^{B}	0.092 ^{B}
RD_{9a}	0.088 ^{E}	0.023 ^{H}
RD_{10a}	0.109 ^{D}	0.077 ^{D}

*mutagen: a - acriflavine, b - ethidium bromide
A, B, C, …. - homogenous groups

After 240 hours the process of glucose fermentation, the budding cells of RD mutants of *Yamadazyma stipitis* ATCC 58376 constituted from 7.9% to 22.7% of total numbers of cells, while part of cells dyed with methylene blue reached values from 26.1% to 39.2%. The best physiological condition after fermentation of glucose - the highest % of budding cells (22.7%) and one of the lowest part of inactive cells (26.9%) - was conspicuous mutant RD_{5A}.

After xylose fermentation part of budding and inactive cells of RD mutants of *Yamadazyma stipitis* ATCC 58376 in total number of cells oscillated respectively from 9.1% (RD_{11A}) to 26.3% (RD_{9A}) and from 17.2% (RD_{9A}) to 34.6% (RD_{4A}). The cells of mutants RD_{7A}, RD_{3B}, RD_{5A} and RD_{6A} were characterized by the best physiological condition after fermentation process.

The budding cells of RD mutants *Pichia stipitis* 1 yeast after the end of glucose fermentation oscillated from 7.8% to 19.4% of total number of cells, while inactive cells - from 13.6% to 29.6%. The best physiological condition was observed in case of mutant RD_{2B}.

The number of cells budding and dyed with methylene blue in total number of cells after xylose fermentation amounted respectively to from 12.8% to 31.9% and from 16.4% to 31.4%. The physiological condition of mutant RD_{3A} after xylose fermentation was the best in this group of yeast, what was expressed by 28.2% of budding cells and 17.1% of inactive cells. The worst physiological condition after fermentation of xylose appeared in strain *Pichia stipitis* 1 and mutant RD_{1B}.

After glucose fermentation process with RD mutants of *Pichia stipitis* 2 strain, budding and inactive cells oscillated respectively from 10.6% (RD_{2B}) to 27.2 % (RD_{1B}) and from 26.4% (RD_{2B}) to 37.6 % (RD_{6A}) of total cells number.

The number of budding cells in total number of cells after xylose fermentation varied between 9.4% and 18.2%, while the of inactive cells – between 13.2 % and 27.2 %. Mutant RD_{6A} was characterized by the biggest number of budding cells, while parental strain *Pichia stipitis* 2 was characterized by the biggest number of inactive cells.

All RD mutants of strains *Yamadazyma stipitis* ATCC 58376 fermented xylose less effectively than glucose, but their physiological condition after the end of xylose fermentation was a little better than after fermentation of glucose.

RD mutants of strain *Pichia stipitis* 1 fermented xylose with lower yield in comparison to glucose, whereas, except for strains RD_{1B}, RD_{2B} and RD_{6A}, in tests with xylose they showed better physiological condition.

All RD mutants of *Pichia stipitis* 2 yeast fermented xylose worse than glucose, whereas their physiological condition after xylose fermentation was better than in case of glucose.

CONCLUSIONS

To sum up, the obtained RD mutants of yeast *Yamadazyma stipitis* ATCC 58376 produced ethanol from glucose with the yield similar to parental strain (87.1÷99.4% yield of parental strain), while xylose - with lower yield (about 16.4÷46.6%) in comparison to strain with full respiratory abilities. RD mutants *Pichia stipitis* 1 and *Pichia stipitis* 2 were characterized by weak yield of glucose fermentation (respectively, about 22.0÷58.8% and 20.0÷58.8% less than parental strains) and xylose (respectively, about 56.7÷76.8% and 40.3÷66.9 % less than parental strains). Apart from that, RD mutation caused in all strains a considerably lower effectiveness of fermentation of xylose in comparison to glucose, even in RD mutants of strain *Yamadazyma stipitis* ATCC 58376, which fermented xylose better than glucose. The physiological condition of RD mutants cells was, however, better after fermentation in xylose containing medium.

Defining a suitable selection marker, differentiating strains and making it possible to select hybrids is an indispensable stage before protoplast fusion. In this work the possibility to obtain RD mutants of xylose-fermenting yeast was examined. Earlier experiments confirmed usefulness of RD mutation as genetic marker of fusion, and RD mutants distillery yeast, wine yeast and brewery yeast, obtained by using acriflavine, in case of ethanol yield did not statistically differ from parental strains, and their physiological conditions after fermentation were better [2, 3]. Besides, it was expected, that respiratory defect of yeast fermenting xylose allowed for partial elimination of fermentation media aeration. However, mutagen concentration– 2 µg acriflavine/ml sufficient to mutation of yeast *Saccharomyces cerevisiae* proved too low to obtain satisfactory degree of mutation of xylose-fermenting yeast, while increasing of acriflavine concentrations to 4 and 6 µg/ml negatively influenced the vitality of microorganisms. Stable RD mutants yeast *Candida shehatae* ATCC 58779 and *Pachysolen tannophilus* ATCC 32691 and *Pachysolen tannophilus* ATCC 60392 were not obtained. Jeffries [7] also informed about similar difficulties with obtaining the stable *petite* mutants of yeast *Candida shehatae*. RD mutants of *Yamadazyma stipitis* ATCC 58376 and *Pichia stipitis* 1 and *Pichia stipitis* 2 yeast achieved considerably lower ethanol yield from xylose than their parental strains, what as a result, eliminates them as potential genetic material donors to increase spectrum of fermentative abilities of traditional distillery yeast for the possibility to pentose utilization. However, in world literature there is not enough information relating to the proprieties of RD mutants of xylose-fermenting yeast. Investigations executed in the present work, regarding different yeast species: *Yamadazyma*, *Pichia*, *Candida* and *Pachysolen*, are an essential contribution to the knowledge of strains established damages in respiratory system.

REFERENCES

1. Campbell I., Duffus J.H. (1988). *Yeast a practical approach*. Oxford: IRL Press.
2. Dziuba E., Kawa-Rygielska J., Chmielewska J. (1997). Wykorzystanie RD mutacji jako markera genetycznego fuzji protoplastów [The use of RD mutation as a genetic marker of protoplasts fusion]. *Materiały XXVIII Sesji Naukowej KTiChŻ PAN, Gdańsk*, 43 [in Polish].
3. Dziuba E., Kawa-Rygielska J., Chmielewska J. (1998). Ocena przydatności drożdży *Saccharomyces cerevisiae* “malt whiskey” do ulepszenia drożdży gorzelniczych [The evaluation of the *Saccharomyces cerevisiae* “malt whiskey” yeast usefulness in the improvement of the distillery yeast]. *Przem. Ferm.*, 8, 31-34 [in Polish].
4. Hahn-Hägerdal B., Jeppsson H., Skoog K., Prior B.A. (1994). Biochemistry and physiology of xylose fermentation by yeasts. *Enzyme Microb. Technol.*, 16, 933-943.
5. Heluane H., Spencer J. F. T., Spencer D.M., de Figueroa L., Callieri D. A. S. (1993). Characterization of hybrids obtained by protoplast fusion, between *Pachysolen tannophilus* and *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.*, 40, 98-100.
6. Ho N.W.Y., Chen Z., Brainard A.P. (1998). Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. *Appl. Environ. Microbiol.*, 64(5), 1852-1859.
7. Jeffries T.W. (1984). Unstable petite and grande variants of *Candida shehatae*. *Biotechnol. Lett.,* 6 (12), 777-782.
8. Jeffries T.W., Kurtzman C.P. (1994). Strain selection, taxonomy, and genetics of xylose-fermenting yeasts. *Enzyme Microb. Technol.*, 16, 922-932.

9. Kordowska-Wiater M., Targoński Z. (1997). Wykorzystanie upośledzonych oddechowo mutantów *Saccharomyces cerevisiae* do łącznej fermentacji glukozy i ksylozy wspólnie z drożdżami *Pichia stipitis* [The use of respiratory deficient mutants of *Saccharomyces cerevisiae* to common fermentation of glucose and xylose together with *Pichia stipitis* yeast]. *Biotechnologia*, 39(4), 94-102 [in Polish].
10. Muralidhar R.V., Panda T. (2000). Fungal protoplast fusion – a revisit. *Bioprocess Engineering*, 22, 429-431.
11. Olsson L., Hahn-Hägerdal B. (1996). Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme Microb. Technol.,* 18, 312-331.
12. Sakai T., Koo K., Saitoh K. (1986). Use the protoplast fusion for the development of rapid starch- fermenting strains of *Saccharomyces diastaticus*. Agric. Biol. Chem., 50 (2), 297-306
13. Shimizu K., Yokomori Y., Kitano K. (1994). Alcoholic beverages: Genetic improvement of yeasts. In ed. by MurookaY., Imanaka T. *Recombinant microbes for industrial and agricultural applications* (543-555). Marcel Dekker.
14. Spencer J.F.T., Bizeau C., Reynolds N., Spencer D.M. (1985). The use of mitochondrial mutants in hybridisation of industrial yeast strains: VI. Characterization of the hybrid, *Saccharomyces diaststicus* x *Saccharomyces* rouxii, obtained by protoplast fusion, and its behaviour in stimuled dough-raising tests. *Curr. Genet.*, 9, 649-652.
15. Spencer J.F.T., Spencer D.M., Bizeau C., Martini A.V., Martini A. (1985). The use of mitochondrial mutants in hybridisation of industrial yeast strains: V. Relative parental contributions to the genomes of interspecific and integeneric yeast hybrids obtained by protoplast fusion, as determined by DNA reassociacion. *Curr. Genet.*, 9, 623-625.
16. Sulo P., Michacakova S., Reiser V. (1992). Construction and properties of K1 type killer wine yeasts. *Biotechnol. Lett.* 14, 55-60.
17. Zaldivar J., Nielsen J., Olsson L. (2001). Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Appl. Microbiol. Bioechnol.* 56, 17-34.

Joanna Chmielewska, Ewelina Dziuba
Department of Food Storage and Technology
Agricultural University of Wroclaw
ul. Norwida 25, 50-375 Wroclaw, Poland
tel.: +48 71 32 05 237, fax: +48 71 32 05 273
e-mail: chmiel@ozi.ar.wroc.pl

Responses to this article, comments are invited and should be submitted within three months of the publication of the article. If accepted for publication, they will be published in the chapter headed ‘Discussions’ in each series and hyperlinked to the article.