Renewable Energy 37 (2012) 197–201

Contents lists available at ScienceDirect

Renewable Energy

journal homepage: www.elsevier.com/locate/renene

Improvement in ethanol production using respiratory deficient phenotype of a wild type yeast *Saccharomyces cerevisiae* ITV-01

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ARTICLE INFO

Article history:
Received 16 February 2011
Accepted 11 June 2011
Available online 7 July 2011

Keywords:
Respiratory deficient
Saccharomyces cerevisiae
Ethanol production

ABSTRACT

An osmotolerant low pH resistant wild type *Saccharomyces cerevisiae* ITV-01 yeast was employed to obtain respiratory deficient (RD) strains in order to improve ethanol production. The respiratory deficient phenotype was induced by exposure to chemical agents such as ethidium bromide, rhodamine, and ethanol. Isolation with physical agents such as temperature and UV radiation (254 nm) exposures was evaluated. 104 strains were tested. Selection criterion was based on increasing ethanol yield. All RD strains had higher ethanol yields (from 0.41 to 0.50 gg^{-1}, corresponding 82–98% theoretical yields) than the wild type yeast (0.40 gg^{-1}). Temperature and ethanol mutant agents caused a reversible phenotype in every case. Screening showed that *S. cerevisiae* ITV-01 RD-B14 (lacking cytochrome c), obtained using bromide ethidium, had the highest alcoholic fermentation efficiency (95%) and 97% viability under the tested conditions (150 gL^{-1} glucose, pH 3.5). The respiratory deficient mutation is of interest as a tool for improving ethanol production.

1. Introduction

Ethanol is an aliphatic alcohol, useful in the chemical, pharmaceutical, beverage and biofuel industries, among others. Therefore, improvement in ethanol production processes is relevant because of its socioeconomical importance. In recent years, new microorganisms with desirable features such as osmotolerance, low pH and ethanol resistance, advantageous to the alcohol fermentation process, have been sought. Previously, it was established that the use of respiratory deficient (RD) strains should be an option for increasing process efficiency [1–4].

RD strain (also known as petite yeasts because the colonies are comparatively small) was discovered by Ephrussi and colleagues in 1949 and it could be defined as those yeasts that show damage at some points in the respiratory chain mainly at cytochrome level (cytochrome a + a_3 and b) [5]. RD mutation occurrence is around 2–3% by the end of alcoholic fermentation, but this phenotype is reversible to the respiratory competent parental yeast in almost all cases [4,6].

RD phenotype is a feature of interest as it causes an increase in alcoholic fermentation efficiency [7], raising the yield close to the theoretical value (0.51 g ethanol g $glucose^{-1}$).

RD mutants are also unable to consume non-fermentable substrates such as glycerol or ethanol, consequently diauxic growth is not present [8]. There are few reports of the effect of RD phenotype on viability, biomass and glycerol production; nevertheless, all of them report an increase in ethanol yield [7–9]. RD strains use for ethanol production has been poorly explored because this type of phenotype could be linked to damage in osmotolerance and ethanol resistance [2–4], the genes related to this feature being part of mitochondrial DNA [10].

However, this phenotype gives the cell the capability to resist several toxic compounds [9,11]. Therefore, the search for osmotolerant RD mutants needs to be performed to find yeast with the best characteristics to improve alcoholic fermentation.

Ethnic *Saccharomyces cerevisiae* ITV-01 yeast was isolated from sugar cane molasses by Ortiz-Zamora et al. [12].

This strain is osmotolerant (250 g glucose L^{-1}) and performs alcoholic fermentation at low pH values (3.0–3.5) with an ethanol yield of 0.405 gg^{-1} and volumetric productivity of 1.83 $gL^{-1}\,h^{-1}$ [13]. The aim of this work was to induce, isolate and select a RD strain from the ethnic *S. cerevisiae* ITV-01 yeast with the best features of the wild type yeast in order to improve ethanol production.

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doi:10.1016/j.renene.2011.06.019

2. Methods

2.1. Microorganism

Wild type *S. cerevisiae* ITV-01 yeast was previously isolated from sugar cane molasses by Ortiz-Zamora et al. [12].

2.2. Induction of respiratory deficient phenotype

RD mutation was induced by exposure to chemical agents: ethidium bromide, rhodamine and ethanol. Additionally, isolation with physical agents such as tempertature and UV radiation (254 nm) exposures was evaluated. The occurrence of spontaneous RD phenotype at the end of several fermentations was also evaluated. The conditions for inducing the RD phenotype were as follows: first the yeast was activated in an Erlenmeyer flask using 150 gL^{-1} glucose based media for 12 h (Preculture and Kinetic media). After that, 6×10^6 viable cell mL^{-1} were inoculated into another flask with 50 mL of culture medium using the same culture medium. The same inoculum was employed in order to induce the RD state using ethanol (20–40% v/v), temperature (60–80 °C), UV radiation (254 nm), rhodamine (10 ppm) and ethidium bromide (10 ppm) for 12 h twice.

Petri dishes were prepared with three culture media: glucose based, glycerol based and differential (these media are mentioned below). Petite colonies were regrown on differential media, and when the pure culture was obtained, they were grown again on the three culture media (conservation, differential and glycerol).

2.3. Culture media

2.3.1. Conservation medium

S. cerevisiae was stored at 4 °C using a culture medium which composition was as follows: glucose, yeast extract and agar (150, 20 and 20 gL^{-1}, respectively).

2.3.2. Preculture and kinetic media

Both media contained: glucose, yeast extract, KH_2PO_4, $(NH_4)_2SO_4$ and $MgSO_4 \cdot 7H_2O$ (150, 2.0, 8.0, 5.0, 1.0 gL^{-1}, respectively). The initial pH was adjusted to 3.5 using 85 %v/v orthophosphoric acid.

The preculture was prepared in a 250 mL Erlenmeyer flask containing 100 mL liquid medium, stirred at 150 rpm. After inoculation, each Erlenmeyer flask was incubated at 30 °C for 12 h. Two precultures were prepared to obtain the inoculum.

2.3.3. Differential medium

A differential medium was used following the method proposed by Nagai [14] in which the RD mutants become coloured. The composition was as follows: glucose, casein peptone, yeast extract, KH_2PO_4, $(NH_4)_2SO_4$ and agar (20, 1.5, 1.5, 1.5, 1.5 and 20 gL^{-1}, respectively). Tryphan blue (18 ppm) and eosyn Y (12 ppm) were added after sterilization under aseptic conditions.

2.3.4. Glycerol based medium

A glycerol based medium containing: glycerol, yeast extract and agar (20, 20 and 20 gL^{-1}, respectively) was employed in order to test the inability of RD mutants to grow on non-fermentable substrates. All culture media were sterilized for 15 min at 121 °C.

2.4. Screening conditions

Duplicate fermentations were carried out in 250 mL Erlenmeyer flasks containing 100 mL medium. The flasks were inoculated with 6×10^6 viable cells mL^{-1} and agitation was fixed at 150 rpm (New Brunswick Scientific classic series C24KC Refrigerated Incubator Shaker Edison NJ, USA).

2.5. Batch culture

Batch cultures were carried out in a Biostat B plus fermentation system (Sartorius Stedim Biochem S.A.) with the media and inoculum mentioned above. The media volume was 2 L, either without air supply (microaerobic) or with an air flow of 0.3 vvm (aerobic).

2.6. Analytical techniques

Yeast growth was measured by direct count using a Thoma Chamber and a correlation optic density (620 nm) against cell dry weight was performed [13].

Viability was assessed by the methylene blue staining method proposed by Lange et al. [15]. The culture medium was centrifuged for 10 min at 10,000 rpm (Eppendorf Centrifuge 5424, Germany), and the supernatant was stored at −20 °C until its analysis. Glucose, glycerol and ethanol were measured by high performance liquid chromatography (Waters 600 TSP Spectra System, Waters, Milford, MA, USA) using a Shodex SH1011 column (8 × 300 mm). Temperature was 45 °C, mobile phase sulphuric acid 10 mM, flow rate 0.6 mL min^{-1} and an Index Refraction detector (Waters 2414, TSP Refracto Monitor V, Waters) was employed. Yeast absolute spectra were performed according to Claisse et al. [16] in order to ascertain which cytochrome was affected by the mutant agent.

3. Results and discussion

3.1. Induction of respiratory deficient mutation

Exposure to chemical mutagenic agents such as ethidium bromide (B) and rhodamine (R) induced 31 and 32 RD strains, respectively. They fitted the previously established criteria for RD mutant strains identification, that is, able to grow on glucose media producing petite colonies, unable to grow on glycerol based media and also coloured on differential media [9,11,14].

Contrary to RD phenotype induction using ethanol or temperature reported by Nagai [14], no RD yeasts could be isolated. This could be due to this agent's inability to irreversibly damage either nuclear or mitochondrial DNA. The use of UV radiation (U) at 254 nm led to the isolation of 38 RD yeasts from *S. cerevisiae* ITV-01. At the end of several fermentations, three spontaneous RD yeasts were also isolated. Finally, a total of 104 RD strains were isolated in this study.

3.2. Screening for ethanol production

In order to select an RD strain with the best features of the wild type yeast, all 104 strains were screened (250 mL Erlenmeyer flask with 100 mL culture media previously mentioned in duplicate). The selection criteria were based on: ethanol yield > 0.405 gg^{-1}; log cell $mL^{-1} > 8.32$; cell viability $> 94\%$; and also, glycerol yield < 0.026 gg^{-1}, according to data previously established by Ortiz-Muñiz et al. [13] for *S. cerevisiae* ITV-01.

Growth was lower in almost all the mutants tested. However, 17 RD strains were able to reach a greater cell density than the respiratory competent yeast. These results suggest that the RD phenotype affect log cell mL^{-1} due to oxidative metabolism depletion. On the other hand, ethanol yield in every mutant case in this study was higher than the wild type yeast (Table 1), similar to what was previously reported by Hutter and Oliver [2]. This increase in ethanol yield identifies the RD state in the mutants tested. RD mutation negatively affects cell resistance under stress

Table 1
Selection of RD mutants of *S. cerevisiae* ITV-01.

Desirable condition	Mutants obtained with	lower value	higher value	Mean ± standard deviation[a]	% mutants	Code of mutants selected
Log (cell mL^{-1})> 8.32	Ethidium bromide	8.01	8.41	8.26 ± 0.14	6.5	B9, B14
	Rhodamine	7.97	8.35	8.16 ± 0.12	6.3	R33, R64, R65, R85, R97, R99
	UV radiation	8.12	8.33	8.21 ± 0.07	5.3	U27, U42, U66, U69, U78, U95
	Spontaneous	8.12	8.22	8.17 ± 0.06	0.0	None
Ethanol yield	Ethidium bromide	0.430	0.505	0.454 ± 0.018	100.0	All RD mutants
> 0.405 gg^{-1}	Rhodamine	0.427	0.480	0.451 ± 0.014	100.0	All RD mutants
	UV radiation	0.415	0.497	0.449 ± 0.020	100.0	All RD mutants
	Spontaneous	0.472	0.506	0.496 ± 0.002	100.0	All RD mutants
Viability > 94%	Ethidium bromide	41	97	69.9 ± 20.9	3.2	B14
	Rhodamine	22	96	50.8 ± 20.5	9.4	R63, R64, R65
	UV radiation	30	95	55.2 ± 20.6	7.9	U17, U28, U80
	Spontaneous	74	97	85.3 ± 13.3	66.7	S1, S2
Glycerol yield	Ethidium bromide	0.024	0.056	0.035 ± 0.019	9.7	B14, B28, B46
< 0.026 gg^{-1}	Rhodamine	0.030	0.069	0.046 ± 0.013	0.0	None
	UV radiation	0.015	0.063	0.044 ± 0.022	13.2	U39, U40, U51, U91, U92
	Spontaneous	0.044	0.057	0.050 ± 0.007	0.0	None

[a] Represent the mean of each strain value.

conditions (150 g glucose L^{-1}, pH 3.5 and the ethanol produced during the fermentation) as indicated by a low cell viability value at the end of fermentations. Only nine RD strains were able to attain higher viability than the wild type.

Almost all RD strains presented an increase in glycerol production (possibly because its physiological function was under stress) thus helping the cell to maintain both water activity and consequently, primary glycolytic enzyme activity [10]. The increase in glycerol production could also be related to the redox imbalance caused by respiratory metabolism saturation due to the lack of cytochromes.

From the data shown in Table 1, B14 petite was selected because it has better parameters for ethanol production such as ethanol yield (0.484 gg^{-1}) and viability (97%). In addition, aggregate formation, shown by B14 petite but not presented in wild type yeast, suggested that RD phenotype also alters cell wall composition and structure, a phenomenon that could be related with flocculation capability and ethanol resistance [3]. In order to corroborate if the RD mutation varied or changed cytochrome composition, absolute spectra of *S. cerevisiae* ITV-01 and the *S. cerevisiae* ITV-01 RD-B14 petite were performed. Absolute spectra analysis (Fig. 1) showed that B14 petite lacks cytochrome c (the electron transporter from complex III to complex IV in the mitochondrial electron transport chain). This result indicates that oxidative metabolism saturation leads to alcoholic fermentation stimulation.

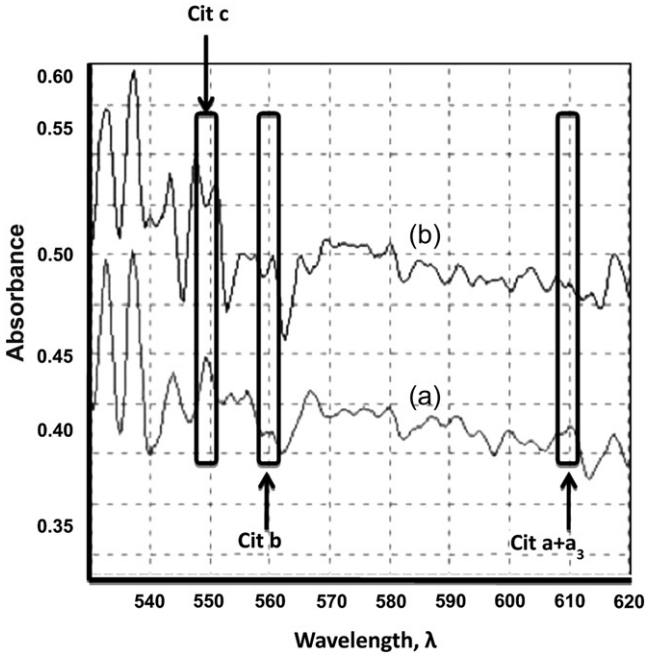

Fig. 1. Absolute spectra of *S. cerevisiae* ITV-01 (a) and RD-B14 (b) petite. Cyt a + a_3, 610 nm; Cyt b, 560 nm; Cyt c, 549 nm.

3.3. Comparative kinetics of S. cerevisiae ITV-01 and the RD-B14 strain

In order to compare the kinetic behaviour of *S. cerevisiae* ITV-01 and the RD-B14 petite, batch cultures were performed under microaerobic (without air supply) and aerobic conditions (0.3 vvm). As shown in Fig. 2, biomass production was lower in RD-B14 petite (4 gL^{-1}) under aerobic conditions as compared with *S. cerevisiae* ITV-01 (6 gL^{-1}). These results are related to the damage in RD-B14 petite oxidative metabolism. No change in RD-B14 petite specific growth rate was observed under microaerobic and aerobic conditions compared with the wild type strain behaviour under the same conditions (Table 2). The lack of oxygen stimulates alcoholic fermentation, although this element is necessary for ergosterol biosynthesis [6], a compound related with plasma membrane stability and thus with ethanol resistance in yeasts.

It is recognized that oxygen stimulates yeast growth and reduces ethanol production, a phenomenon illustrated in the results shown in Table 2. However, ethanol concentration under aerobic conditions

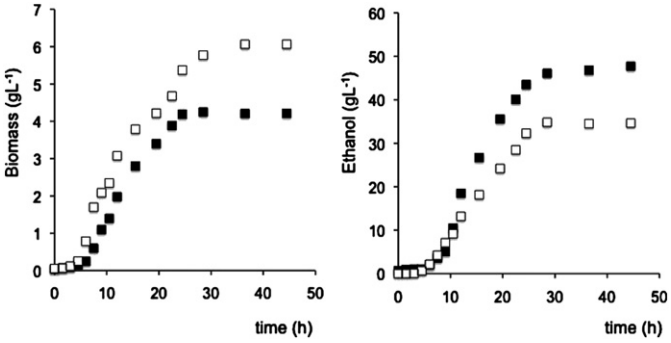

Fig. 2. Growth and ethanol production of *S. cerevisiae* ITV-01 and B14 petite under aerobic conditions: *S. cerevisiae* ITV-01 (□), B14 petite (■).

Table 2
Fermentation parameters of *S. cerevisiae* ITV-01 and B14 petite under microaerobic and aerobic conditions.

Parameters	Microaerobic		Aerobic	
	S. cerevisiae ITV-01	B14 petite	*S. cerevisiae* ITV-01	B14 petite
Ye/s (g ethanol g glucose^{-1})	0.405	0.484	0.268	0.369
Yx/s (g biomass g glucose^{-1})	0.037	0.031	0.046	0.034
Gp (glycerol production, gL^{-1})	4.43	3.68	6.50	4.46
Ep (ethanol production, gL^{-1})	60.8	72.6	35.0	47.0
Ye/x (g ethanol g biomass^{-1})	10.94	17.85	5.78	10.81
μ (specific growth rate, h^{-1})	0.31	0.32	0.41	0.34
Ve (g ethanol g biomass^{-1} h^{-1})	1.72	1.84	1.13	2.39
Qe (g ethanol L^{-1} h^{-1})	1.46	1.63	1.24	1.62
Efficiency (%)	79.4	94.9	52.5	72.3

for the RD strain was higher than in the wild type yeast (47 and 35 gL^{-1}, respectively). These results contrast with those previously reported by Bacila and Horii [4] because under test conditions, RD strain ethanol production was always greater than for the wild type yeast. The increase in RD-B14 petite specific ethanol production rate and specific yield and the decrease of these values in *S. cerevisiae* ITV-01 indicate a major repression by the Crabtree effect (stimulation of alcoholic fermentation under aerobic conditions) over the Pasteur effect (inhibition of alcoholic fermentation under aerobic conditions); this phenomena was previously reported for RD yeasts [7,17]. In addition, alcoholic fermentation efficiency (obtained yield/theoretical yield) in *S. cerevisiae* ITV-01 was 79.4%, whereas for RD-B14 petite it was 94.9%, showing that the RD mutation improves alcoholic fermentation performance. However, the results shown in Table 2 illustrate that both the wild type and the RD strain have similar values in ethanol productivity (Qe = 1.46 gL^{-1} h^{-1} and 1.63 gL^{-1} h^{-1} respectively) and specific ethanol production rate (Ve = 1.72 gg^{-1} h^{-1} and 1.84 gg^{-1} h^{-1} respectively) under microaerobic conditions employed in the industrial process for ethanol production. These results are in agreement with those previously reported [18]. Therefore the main advantage of using RD strains must be to increase ethanol yield (alcoholic fermentation efficiency), diminishing biomass production by 33% (from 6 to 4 g biomass L^{-1}), but keeping Qe and Ve at values similar to the wild type yeast under microaerobic conditions.

Glycerol production was lower for B14 petite than for the wild type yeast under microaerobic and aerobic conditions (3.68, 4.43 and 4.46, 6.50 g glycerol L^{-1}, respectively). These results concur with those previously reported [19], where optimal oxygen availability decreased glycerol production almost fourfold. It could be inferred that when using the respiratory deficient phenotype, the oxygen supply must be increased in order to diminish the redox imbalance caused by cytochrome lack; this would also increase glycerol production under aerobic conditions. Further studies must be carried out in order to establish the best conditions for ethanol production using *S. cerevisiae* ITV-01 and its RD-B14 mutation.

4. Conclusions

RD phenotype in *S. cerevisiae* ITV-01 is induced by ethidium bromide, rhodamine, UV radiation and also occurs spontaneously at the end of fermentation. However, temperature and ethanol as agents for inducing RD phenotype produced a reversible RD state. The highest alcoholic fermentation efficiency (94.9%) and 97% viability under tested conditions were observed in the RD-B14 petite, compared to the wild type yeast (79.4%). This RD-B14 petite phenotype is due to its lack of cytochrome c. The differences found in specific ethanol yield and specific growth rate under microaerobic and aerobic conditions indicate that the RD phenotype in *S. cerevisiae* ITV-01 is due to electron transport deficiency, which causes pyruvate metabolism saturation related to the occurrence of the Crabtree effect in yeasts. Furthermore, the study of this phenotype would be helpful in understanding the physiological changes due to metabolic pathway regulation in yeasts caused by the presence of oxygen, as well as the causes of the Crabtree effect. RD state leads to an increased fermentative capability in *S. cerevisiae* ITV-01 and should be considered as a powerful tool for improving the ethanol production.

Acknowledgements

Authors acknowledged the economic support from the National Council of Science and Technology, Mexico (CONACYT, scholarship 45161 and project 128052) and the critical reading of Patricia Hayward Jones MSc. and Dulce María Barradas Dermitz MSc.

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