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Selection and Adaptation of *Saccharomyces cerevisae* to Increased Ethanol Tolerance and Production

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Abstract

A total of 24 yeast strains were tested for their capacity to produce ethanol, and of these, 8 were characterized by the best ethanol yields (73.11–81.78%). The most active mutant *Saccharomyces cerevisiae* ER-A, resistant to ethanol stress, was characterized by high resistance to acidic (pH 1.0 and 2.0), oxidative (1 and 2% of H_2O_2), and high temperature (45 and 52°C) stresses. During cultivation under all stress conditions, the mutants showed a considerably increased viability ranging widely from about 1.04 to 3.94-fold in comparison with the parent strain *S. cerevisiae* ER. At an initial sucrose concentration of 150 g/l in basal medium A containing yeast extract and mineral salts, at 30°C and within 72 h, the most active strain, *S. cerevisiae* ER-A, reached an ethanol concentration of 80 g/l, ethanol productivity of 1.1 g/l/h, and an ethanol yield (% of theoretical) of 99.13. Those values were significantly higher in comparison with parent strain (ethanol concentration 71 g/l and productivity of 0,99 g/l/h). The present study seems to confirm the high effectiveness of selection of ethanol-resistant yeast strains by adaptation to high ethanol concentrations, for increased ethanol production.

Key words: adaptation to high ethanol concentration, ethanol tolerance, fermentation, yeast

Introduction

Bio-ethanol production by yeast is a growing industry due to energy and environmental demands (Schubert, 2006). *Saccharomyces cerevisiae* and related yeast species have been extensively used in fermentation, wine-making, sake-making and brewing processes. Successful performance of alcoholic fermentations, however depends on the ability of the yeast strains used to cope with a number of stress factors occurring during the process (Van Uden, 1985; Viegas *et al.*, 1989; Hirasawa *et al.*, 2007), including osmotic pressure imposed by the initial high sugar concentration and stress induced by fermentation end-products or sub-products such as ethanol or acetate. Among these, the stress induced by increasing amounts of ethanol, accumulating to toxic concentrations during ethanol fermentation, is the major factor responsible for reduced ethanol production yields and, ultimately, for stuck fermentations (Gibson *et al.*, 2007). Thus, yeast strains that can endure stress imposed by high ethanol concentrations are highly desirable. Throughout the years many efforts have been made to characterize the mechanisms underlying ethanol stress tolerance, aiming to increase ethanol productivity (Van Uden, 1985; You *et al.*, 2003; Alper *et al.*, 2006; Hirasawa *et al.*, 2007). To overcome fermentation problems, sophisticated refinements of fermentation processes, involving extractive fermentation (Jones *et al.*, 1993; Da Silva *et al.*, 1999), cell immobilization (de Vasconcelos *et al.*, 2004; Verbelen *et al.*, 2006), and recycling or retention by membranes (Nishiwaki and Dunn, 1998; Wang and Lin, 2010), were employed with a view to obtaining a large quantity of fermenting biomass as well as removing the inhibitory ethanol product. Successful engineering of yeast transcription machinery for this purpose was also reported (Alper *et al.*, 2006).

The present study was conducted to select the best ethanol-producing yeast strains from our collection, to improve yeast fermentation performance by adaptation, and to optimize the conditions for alcohol production from sucrose. There are, to our knowledge, only a few studies that describe the creation of ethanol-tolerant *S. cerevisiae* mutants using adaptation and ethanol stress as the selection pressure (Brown *et al.*, 1982; Remize *et al.*, 1999; Stanley *et al.*, 2010).

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Experimental

Material and Methods

Microorganisms and media. The strains of the yeast listed in Table I were maintained at 4°C on malt agar slants. For inoculum preparation selected strains were cultivated on a growth medium A containing glucose, 2%; bactopepton, 2% and yeast extract, 1%. Fermentations were performed using a basal medium A containing: sucrose, 15.0%; yeast extract, 1.0%; $(NH_4)_2SO_4$, 0.3% and KH_2PO_4, 0.1%.

Adaptation for increased ethanol production. Enrichments for increased ethanol production were carried out according to the method of Dinh *et al.*, (2008) with some modifications. The cultivation of the *S. cerevisiae* ER strain was carried out in malt medium containing ethanol and then the culture was transferred to a fresh medium containing the same ethanol concentration. Adapted cultures were grown in culture tubes (18 by 150 mm) containing 10 ml of that medium and were incubated at 30°C without agitation. After that, the culture was transferred to a medium containing a higher ethanol concentration, followed by repetitive cultivations. The initial ethanol concentration was set at 5.0% (w/v) and it was changed gradually from 6.0 to 15.0%. New derivatives of *S. cerevisiae* were isolated from the adapted cultures after 10 months of serial transfers into media with high concentrations of ethanol. At the end of this period, two clones were selected for further study; these clones were designated as strain ER-A and ER-M.

The adaptation of the yeast was evaluated by measuring the optical density of the culture at OD_{600}. Viability in ethanol was determined by maintaining yeast cells of *S. cerevisiae* ER in malt medium supplemented with 5–15% ethanol for 48 h at 30°C, plating them on malt agar plate, and subsequently counting the number of colonies formed. Control cultures were maintained in the same medium without ethanol.

Inoculum preparation. For the preparation of inoculum, yeast strains were transferred from agar

Table I
Screening yeast strains for efficient ethanol production

Strain	Sucrose concentration (%)					
	15	40	15	40	15	40
	Catalase activity (U)		Ethanol (% w/v)[a]		Ethanol yield (% of theoretical)	
Candida shaetaceae ATCC 22–994	8.67	6.61	0.70	0.40	8.67	1.86
Candida utilis CCY 29–38–18	8.70	7.13	2.00	0.70	24.78	3.25
Kluyveromyces fragilis IPF	8.07	10.00	5.00	3.00	61.96	13.94
Kluyveromyces marxianus CCY 50–2–1	4.38	4.69	3.10	4.30	38.41	19.98
[1]*Saccharomyces bayanus* S-21	4.25	1.89	6.60	7.60	81.78	35.32
[1]*Saccharomyces owiformis*	3.78	5.34	6.00	7.00	74.35	32.53
[2]*Saccharomyces carlsbergensis* FD	4.31	4.48	6.48	6.49	80.30	30.16
[3]*Saccharomyces cerevisiae* Anker	2.58	6.03	5.80	8.10	71.87	37.64
Saccharomyces cerevisiae A364A	1.83	2.08	3.80	2.80	47.09	13.01
Saccharomyces cerevisiae DBY 747	6.81	6.64	6.40	6.80	79.30	31.60
[4]*Saccharomyces cerevisiae* ER	3.78	3.08	6.60	9.75	81.78	45.31
[4]*Saccharomyces cerevisiae* GM	5.20	9.04	5.80	6.00	71.87	27.88
[3]*Saccharomyces cerevisiae* Hammer	3.22	6.75	5.80	9.20	71.87	42.75
[1]*Saccharomyces cerevisiae* JA	8.41	5.34	5.90	7.20	73.11	33.46
[2]*Saccharomyces cerevisiae* PG	4.06	5.09	6.00	6.80	74.35	31.60
[1]*Saccharomyces cerevisiae* J	5.81	4.84	1.10	2.40	13.63	11.15
[1]*Saccharomyces elipsoideus* Sz.o.	3.81	4.29	6.48	5.64	80.30	26.21
Saccharomyces fragilis II 11	4.71	4.90	5.00	2.20	61.96	10.22
Saccharomyces fragilis 11–54	9.07	7.72	3.80	6.20	47.09	28.81
Saccharomyces fragilis S-24	9.84	5.91	3.10	3.20	38.41	14.87
Saccharomyces fragilis S-25	6.88	8.05	5.10	6.20	63.20	28.81
[1]*Saccharomyces mellis* 1	3.71	3.78	0.70	0.80	8.67	3.72
Saccharomyces muciparus CCM 21–25–1	8.54	7.72	4.10	3.00	50.80	13.94
[1]*Saccharomyces rouxii*	7.53	8.71	0.60	0.20	7.43	0.93

The strains were incubated in 50 ml conical flasks, each containing 20 ml of basal medium A containing 15% or 40% of sucrose during 72 h.

[a] Values are averages of 3 replicate determinations with standard deviations of < ± 5%

[1] Wine yeasts; [2] Brewing yeasts; [3] Baker's yeast; [4] Distillery yeast

slants into 50-ml Erlenmeyer flasks containing 10 ml of sterile liquid growth medium A, which were cultured at 30°C on a rotary shaker (Shaker Orbit The LAB-LINE Instruments Inc, Melrose Park, Illinois, USA) at 150 rpm for 24 h.

Ethanol fermentations. Fermentations with the selected strain were conducted in 300-ml Erlenmeyer flasks containing 100 ml of basal medium A (pH 5.5). The flasks were inoculated with 10% v/v seed culture. Fermentations were carried out for 72 h at 30°C (if not indicated otherwise) in a shaker at 90 rpm. Ethanol evaporation was prevented by rubber stoppers, with fermentative tubes filled with 50% H_2SO_4. The fermentation parameters were corrected by ethanol, sucrose and biomass withdrawn during sampling. Overall biomass as well as ethanol yields and sucrose consumption were calculated from end-of-batch data, where the peak ethanol concentration was recorded. Other methodological details are given in tables and figures.

Assays. Biomass was estimated from optical density at 600 nm. Dry mass was calculated by referring to a standard curve of cell mass versus absorbance (Hughest *et al.*, 1984). Ethanol was quantified using the Gonchar *et al.* (2001) method in own modification using o-dianisidine instead of 3,3·5,5·-tetramethylbenzidine (TMB) as chromogen.

Extracellular catalase activity was measured spectrophotometrically by observing the decrease in light absorption at 525 nm during decomposition of H_2O_2 by the enzyme (Fiedurek and Gromada, 1997). One unit (U) of catalase activity was defined as the amount of enzyme catalysing the decomposition of 1 μmol hydrogen peroxide per min at 30°C. Fermentations were performed in 2 replicate cultures, and analyses were carried out in duplicate. The data given here are the averages of the measurements.

Measurement of respiration. Cell suspensions, prepared as described above, were used to measure the rate of yeast respiration at various medium pH, either in the absence or in the presence of ethanol. Other methodological details are given in the tables and figures. Oxygen concentration in the culture medium was measured by a polarographic dissolved oxygen sensor (Ingold, CH Industrie Nord, Urdorf, Switzerland). The readings were expressed as percentage of the initial level of saturation (100%).

Results

Production of ethanol during fermentation is limited by the inability of yeast to grow at high ethanol levels, which is why a great deal of effort has been devoted to creating yeast strains that would tolerate high ethanol levels, and be able to continue the fermentation to produce higher concentrations of alcohol. The development of such strains would have the major advantage of saving the energy involved in distilling and refining ethanol.

A total of 24 yeast strains were tested for their capacity to produce ethanol, and of these, 8 were characterized by the best ethanol yields (73.11–81.78%). Ethanol production, catalase activity, and ethanol yield were monitored on synthetic medium A. The effects of increasing sucrose concentration from 15 to 40% on ethanol yield, catalase activity and biomass of the yeast strains were examined. Along with the increase in sucrose concentration from 15 to 40%, a decrease in biomass and ethanol yield was observed. On the other hand, the increase enhanced extracellular catalase production, probably as an effect of stress conditions. Among the 24 strains, 13 (54.2%) were characterized by high catalase activity when grown on a medium with a high sucrose concentration (40%) (Table I). The increase in sucrose concentration in the medium (from 15 to 40%) caused a significant (1.02 to 3-fold) reduction in biomass production.

For further selection an industrial strain of *Saccharomyces cerevisiae* ER characterized by high ethanol tolerance, higher cell viability especially during "very high gravity" fermentation, and working under a wide range of temperatures (35–40°C) was used. This strain was used for preparation of inocula for adaptation with high concentrations of ethanol (5 to 15%) in order to select ethanol-tolerant yeast. New derivatives of *S. cerevisiae* were isolated from adapted cultures after 10 months of serial transfers. During this period about 120 subsequent transfers were performed. At the end of this period, a two clones were selected from enrichment for further study; those clones, designated as strain *S. cerevisiae*: ER-A and ER-M, were able to grown at 15% of ethanol in the medium (data not shown). When subjected to a stepwise increase in ethanol concentration with repetitive cultivations, the yeast cells *S. cerevisiae* ER-A adapted to the high ethanol concentration showed better biomass accumulation in the medium containing the same ethanol concentration, in comparison to the cells of the parent strain (Table II). This strain was used for further study.

The most active mutant, *S. cerevisiae* ER-A, resistant to ethanol stress, was characterized by high resistance to acidic (pH 1.0 and 2.0), oxidative (1 and 2% of H_2O_2) and high temperature (45 and 52°C) stresses. The viability of mutants during cultivation under all the mentioned stress conditions increased about 1.04 to 3,94-fold in comparison with the parent strain *S. cerevisiae* ER. It is worth noting, that mutant of *S. cerevisiae* ER-A resistant to ethanol stress, generally showed a better adaptation to higher ethanol concentration, as expressed by the increased (about 4-fold) viability at 20% of ethanol in comparison to its 10%

Table II
Effect of ethanol concentration on biomass production

Ethanol (% w/v)	Dry matter (g/l)[a] after		Relative to the parent-type (-fold)	
	24 h	48 h	24 h	48 h
Saccharomyces cerevisiae ER (parent)				
Control (none)	2.33	3.04		
11	1.10	1.67		
12	0.58	1.11		
13	0.41	0.63		
14				
15	0.19	0.22		
Saccharomyces cerevisiae ER-A				
Control (none)	2.42	2.74	1.04	0.90
11	1.35	1.67	1.23	1.00
12	0.65	1.24	1.12	1.12
13	0.53	0.87	1.29	1.38
14	0.44	0.77	1.42	1.64
15	0.30	0.36	1.58	1.64

The strains were incubated in 50 ml conical flasks, each containing 20 ml of basal medium A with ethanol (11–15%) during 24–48 h.
[a] Values are averages of 6 replicate determinations with standard deviations of < ± 6%

Table III
Effect of abiotic stresses on surviving cells of *Saccharomyces cerevisiae* ER

Stress conditions	Surviving cells (%)[a]		Relative to the wild-type (-fold)
	Parent	ER-A	
Low pH (2.0)	81.99	93.70	1.14
Low pH (1.0)	3.85	7.14	1.85
Oxidative stress (1% H_2O_2)	80.90	100.0	1.24
Oxidative stress (2% H_2O_2)	72.0	96.50	1.34
High temperature (45°C)	47.59	69.85	1.46
High temperature (52°C)	26.34	43.17	1.64
Ethanol (10%)	89.25	90.37	1.04
Ethanol (15%)	61.02	77.93	1.28
Ethanol (20%)	10.00	39.40	3.94

The strains were incubated in 50 ml conical flasks, each containing 20 ml of basal medium during 24 h of stress conditions. Low pH media were obtained by adding 0.1 M HC1.
[a] Values are averages of 4 replicate determinations with standard deviations of < ± 4%

concentration (Table III). A similar trend was also observed for oxidative (1 and 2% of H_2O_2) and high temperature (45 and 52°C) stress, when higher numbers of cells survived in more drastic stress conditions.

Measurements of oxygen consumption at 30°C and pH 4.5 showed that ethanol inhibited the respiration rate of yeast. In the absence of added ethanol, the oxygen concentration decreased gradually over the first 15 min to the level of about 6%. When the experiment was repeated in the presence of 5% (w/v) ethanol, the respiration rate of the yeast cells was markedly inhibited, and the oxygen concentration fell more slowly, reaching 10.7% after 20 min. The higher concentration (10%) of ethanol significantly reduced oxygen consumption; in these conditions, more than half of the initial dissolved oxygen content remained

Table IV
Effect of externally added ethanol at concentration 0–15% on CO_2 and biomass production by parent and adapted strain of *Saccharomyces cerevisiae* MR-A

Ethanol (% w/v)	Relative metabolic rate (%)[a, b]		Dry matter (g/l)[b]
	Mixing (90 rpm)	Mixing (180 rpm)	
Saccharomyces cerevisiae ER (parent)			
Control (none)	39.13	73.90	2.33
5	21.74	39.13	1.73
10	4.35	4.35	1.36
11	0	4.30	1.10
12	0	0	0.58
13	0	0	0.39
14	0	0	0.28
15	0	0	0.19
Saccharomyces cerevisiae ER-A			
Control (none)	43.48	100.00	2.46
5	30.43	52.17	2.25
10	5.43	13.44	1.50
11	4.43	13.00	1.35
12	0	4.35	0.70
13	0	0	0.53
14	0	0	0.44
15	0	0	0.34

[a] Relative metabolic rate (%) was calculated by counting the number of bubbles of CO_2 released from fermentative tubes during 60 min. Maximal amount of bubbles formed during culture of the adapted strain ER-A was defined as 100%. The strains were incubated in 50 ml conical flasks, each containing 20 ml of basal medium A with ethanol (5–15%) during 24 h
[b] Values are averages of 6 replicate determinations with standard deviations of < ± 5%

Table V
Effect of $(NH_4)_2SO_4$ concentration on ethanol production

Medium	Ethanol (% w/v)	Ethanol yield (% of theoretical)	Dry matter (g/l)
Saccharomyces cerevisiae ER (parent)[a]			
Basal medium A	6.30	78.06	5.38
Modified basal medium A with $(NH_4)_2SO_4$ – 1,0%	7.10	87.98	6.75
Saccharomyces cerevisiae ER-A [a]			
Basal medium A	7.20	89.22	5.04
Modified basal medium A with $(NH_4)_2SO_4$ – 1,0%	8.00	99.13	6.23

Fermentation conditions: inoculation: 2×10^7 cells/ml, time fermentation 72 h at 30°C in shaker 90 rpm. The strain was incubated in 50 ml conical flasks, each containing 20 ml of basal medium A containing 0.3 and 1.0% of with $(NH_4)_2SO_4$.
[a] Values are averages of 6 replicate determinations with standard deviations of < ± 5%

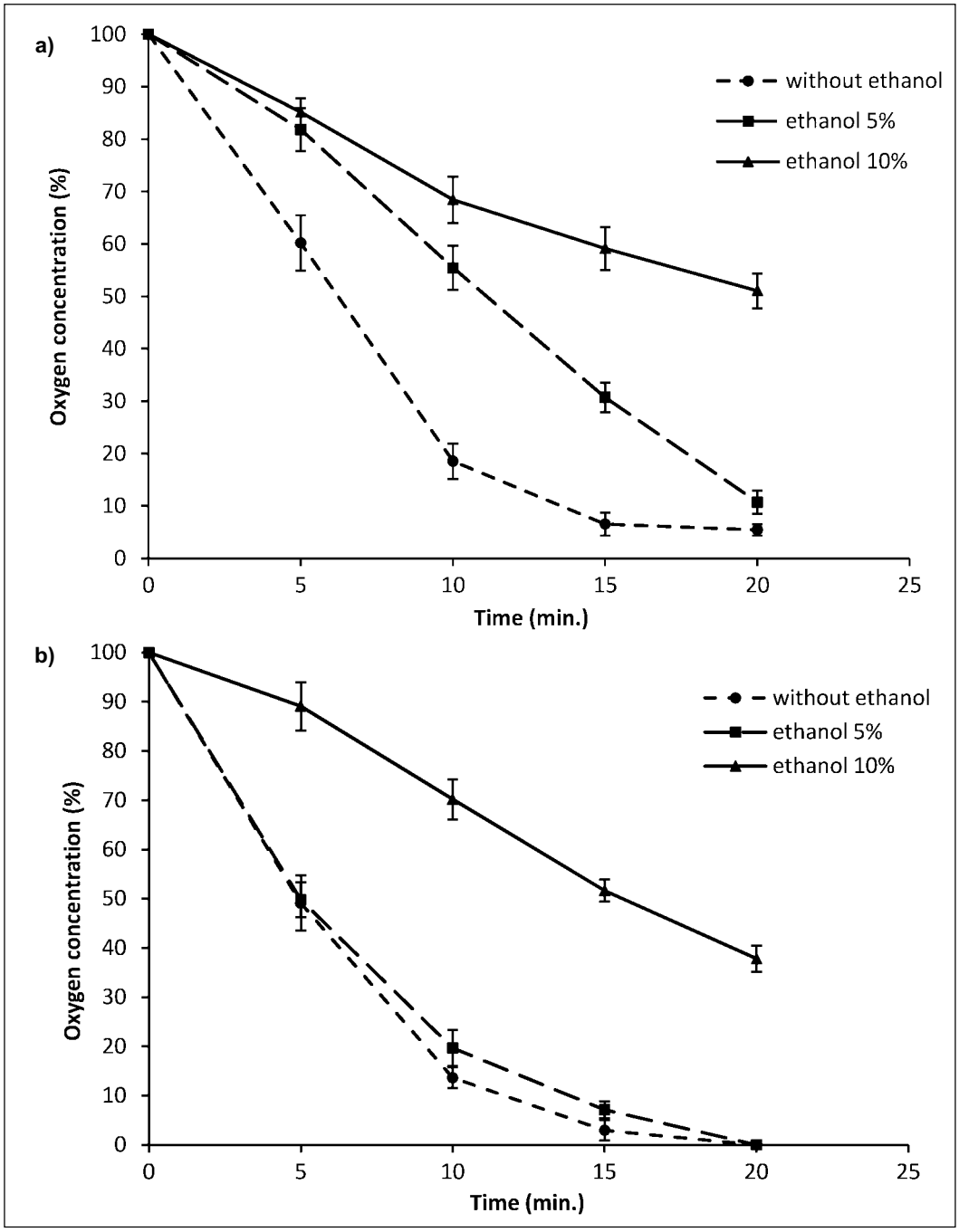

Fig. 1. Effect of ethanol concentration in the medium on the oxygen consumption of *Saccharomyces cerevisiae* ER strain (a) and *Saccharomyces cerevisiae* ER-A strain (b).
The values shown represent the mean of three experiments. Error bars represent standard deviations.

in the medium after 20 min from the beginning of the experiment (Fig. 1a). The adapted cells of *S. cerevisiae* ER-A consumed more oxygen than the parent strain in media with all the tested ethanol concentrations (Fig. 1b). After 20 min of respiration, the levels of oxygen concentration in media with 5% and 10% ethanol were lower by 10.7% and 13.2%, respectively, as compared with values for the parent strain.

The effect of 5–15% ethanol externally added to basal medium A on CO_2 production by the parent and the adapted strain of *S. cerevisiae* ER-A was examined. In comparison with the parent strain, the metabolic activity of adapted cells of *S. cerevisiae* ER-A showed higher resistance to inhibition by ethanol when the cells were grown with 5 and 10% (w/v) ethanol. The higher concentration (11%) of ethanol completely inhibited metabolic activity of the parent strain when culture was carried out in a shaker at 90 rpm, and adapted cells showed insignificant activity in these conditions. Under a higher mixing rate (180 rpm), at 11% of ethanol, adapted cells of *S. cerevisiae* ER-A were characterized by an about 3-fold higher

metabolic activity than the parent strain. A further increase in ethanol concentration in basal medium A to 12% completely inhibited metabolic activity of the parent strain, and the adapted strain showed very small this activity in these conditions (Table IV).

Table V compares ethanol production by the parental and the mutant strain of *S. cerevisiae* ER-A in basal and modified medium A. The highest ethanol yields for the two strains were obtained on modified medium A, containing 15% of sucrose, 1% of yeast extract, 1% of $(NH_4)_2SO_4$ and 1% of KH_2PO_4, using a very simple fermentation system (shake flask). Considerably better results were achieved for mutant *S. cerevisiae* ER-A, which was characterized by an ethanol yield of 99.13% and an ethanol concentration of 8.0%; the respective values for the parental strain were 87.98 % and 7.1%.

Discussion

Tolerance to high ethanol and sucrose concentrations is an important property of industrial microorganisms. The accumulation of ethanol during cultivation causes stress to yeast cells, leading to a decrease in cell growth and production of target products. Thus, understanding the process of adaptation of yeast to high ethanol concentrations is important as it may lead to the construction of yeast strains able to grow well at high ethanol concentrations. Such ethanol-tolerant yeasts are highly desirable for the production of useful compounds. Improving ethanol tolerance in yeast should, therefore, reduce the impact of ethanol toxicity on fermentation performance (Dinh *et al.*, 2008; Stanley *et al.*, 2010).

Stanley *et al.* (2010) obtained ethanol-tolerant yeast mutants by subjecting mutagenised and non-mutagenised populations of *S. cerevisiae* W303-1A to adaptive evolution using ethanol stress as a selection pressure. Mutants CM1 (chemically mutagenised) and SM1 (spontaneous) had increased acclimation and growth rates when cultivated in sub-lethal ethanol concentrations, and their survivability in lethal ethanol concentrations was considerably improved compared with the parent strain. Those authors suggested that the increased ethanol tolerance of the mutants was due to their elevated glycerol production rates and the potential of these to increase the ratio of oxidised and reduced forms of nicotinamide adenine dinucleotide (NAD^+/NADH) in an ethanol-compromised cell, stimulating glycolytic activity.

The viability of the adapted *S. cerevisiae* ER-A was always higher than for the parental strain, for all the stress conditions used (Table III). For example, the viable population (expressed as a percentage of the initial population) of the ER- culture after 24 h in 20% (w/v) ethanol was 39.40%, respectively, compared with 10.0% for the parent. Similar viability characterized SM1 and CM1 cultures obtained by Stanley *et al.* (2010) under lethal ethanol stress conditions (12% (w/v) ethanol, after 12 h) – 52% and 44%, respectively, compared with 5% for the parent.

Some researchers have analyzed phenomena associated with adaptation of yeast cells to high ethanol concentrations. Lloyd *et al.* (1993) found that yeast previously grown in the presence of 5% ethanol could grow in the medium containing 10% ethanol, whereas yeast inoculated directly into a medium containing 10% ethanol failed to grow. Ismail and Ali (1971) reported that no increase in the tolerance of yeast to a high ethanol concentration was observed after ten successive transfers to an environment containing a high ethanol concentration. Therefore, it is expected that exposing yeast cells to a stepwise increase in the level of ethanol stress should be effective for obtaining ethanol-tolerant yeast strains.

The results presented above confirmed that an adapted strain resistant to ethanol stress generally showed better adaptation to other stress conditions, as expressed by the increased survival of the mutant of *S. cerevisiae* ER-A during cultivation under acidic (pH 1.0 and 2.0), oxidative (1 and 2% of H_2O_2), and high temperature (45 and 52°C) stresses. It is worth noting that in the more drastic stress conditions, the ethanol-tolerant mutant was characterized by a higher survival rate. This is in accordance with data presented by Ogawa *et al.* (2000), who showed that several genes were highly expressed only in the ethanol-tolerant mutant but not in the parent strain. The ethanol-tolerant mutant also exhibited resistance to other stresses including heat, high osmolarity, and oxidative stress in addition to ethanol tolerance. These results indicate that the mutant exhibits multiple stress tolerances due to elevated expression of stress-responsive genes, resulting in accumulation of high amounts of stress protective substances such as catalase, glycerol, and trehalose (Ogawa *et al.*, 2000). The ability of one stress condition to provide protection against other stresses is referred to as cross-protection. Several studies have shown that adaptation to acid stress confers resistance to a wide range of stress conditions including heat, salt, crystal violet, and polymyxin B (Lee *et al.*, 1995; Bearson *et al.*, 1997). However, adaptation to other stresses does not typically induce significant acid tolerance. This implies that acid exposure may be treated by microorganisms as a more general stress indicator, whereas salt and H_2O_2 may be more specific stress signals.

A number of specific selection schemes have been elaborated to improve the biosynthetic capacity of production strains. Thus the acid tolerance of *Leuconostoc oenos* was examined in cells surviving at pH

2.6, which is lower than the acid limit of growth (about pH 3.0). The acid-resistant mutant *L. oenos*, was found to be able to grow in acidic media and characterized by a high H^+–ATPase activity at low pH. Such strains may be an important part of the technology of modern commercial wine production (Drici-Cachon *et al.*, 1996). Accumulation of a large amounts of metabolic end-products during the fermentation period, especially in case of industrial amino acid fermentation, builds up a high osmotic strength which affects both growth and production. Enhanced l-treonine production by salt tolerant mutants of *E. coli* was achieved (Drici-Cachon *et al.*, 1996). Some mutants have been described in *E. coli* which are more resistant to cell lysis in the presence of ethanol (Fried and Novick 1973; Ingram *et al.*, 1980). In this respect, our results obtained in ethanol production can be compared with data provided by Ortiz-Zamora *et al.* (2008), who isolated and selected yeast strains from alcoholic fermentations of natural sources. These strains were exposed several times to high concentrations of glucose and ethanol in order to select ethanol- and glucose-tolerant yeast; 10 were obtained that adapted best to these conditions. Some of these strains demonstrated the highest adaptation to both ethanol (5–7% w/v) and glucose (20% w/v). The maximum yield obtained was 0.46 g/g (90% theoretical yield) in a 20-L bioreactor with cane molasses.

Araque *et al.* (2008) selected thermotolerant yeast strains *Saccharomyces cerevisiae* for bioethanol production, which were able to grow and ferment glucose in the temperature range 35–45°C. All the strains grew (in agar plates) at 35 and 40°C, only two strains grew at 42°C, and no strain grew at 45°C. Glucose-to-ethanol conversion yield was between 50% and 80% of the theoretical value. The ethanol yields by SSF using the selected strain were higher than those obtained using the control yeast.

The selected strain, *S. cerevisiae* ER-A, showed an ability to grow and ferment sucrose at ethanol concentrations in the medium of 15 and 12% (w/v), respectively (Tables II and IV). Its resistance to ethanol, externally added to the medium, was significantly higher than for the parent strain. An increase in the rate of mixing from 90 to 180 rpm correlated with a simultaneous increase in the relative fermentation rate (%) both for the parent and the adapted strain, probably as an effect of a higher mass transfer. The effect of ethanol on yeast growth and fermentation has been studied by Brown *et al.* (1981).These authors showed complex kinetics which resulted from both an inhibition of the growth rate itself and also a reduction in cell viability. The growth and viability effects had different inhibition constants. Contrary to our data, ethanol was less inhibitory toward fermentation than toward growth in sake yeast.

Some data suggest that an improvement in ethanol tolerance leads to an increase in both ethanol production rate and the total amount of ethanol produced (Jiménez and Benítez, 1988). The adapted *S. cerevisiae* ER-A reached an ethanol concentration of 80 g/l, an ethanol productivity of 1.1 g/l/h, and an ethanol yield (% of theoretical) 99.13. Those values were significantly higher in comparison with the parent strain (ethanol concentration of 72.9 g/l and productivity of 1,01 g/l/h).

The studies presented above seem to confirm the high effectiveness of selection of resistant yeast strains by adaptation to high ethanol concentrations for increased ethanol production. Additionally, better adaptation of these mutants to abiotic stresses can affect yeast growth and ethanol productivity. The advantage gained in direct screening is to reduce in a very specific way the number of cultures isolated from the plates, which would normally require testing of productivity *via* shake flask cultures. This is a significant contribution to make screening of ethanol-producing yeast more efficient. The ethanol tolerant strain was stable in the subsequent subcultures in the absence of stress during 6 months. It can be concluded from the present results that the adapted strain *S. cerevisiae* ER-A showed, at this stage of our studies, a moderate fermentation activity, which gave reasonable ethanol yields from sucrose. Further improvements to the isolated yeast strain and the growth conditions are necessary to utilize the strain for larger-scale fermentation.

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