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ORIGINAL PAPER

A comparison of stress tolerance in YPD and industrial lignocellulose-based medium among industrial and laboratory yeast strains

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Abstract In general, it is believed that fermentation by yeast under harsh industrial conditions, especially if substrates such as wood hydrolysate or lignocellulosic substrates are used, requires the use of so-called industrial strains. In order to check whether this is always true, a comparison of performance was made using two industrial strains and four commonly used laboratory strains, the haploid and diploid versions of CEN-PK and X2180, under industrially relevant stress conditions. The industrial strains were a Swedish commercial baker's yeast strain and a strain previously isolated from an industrial bioethanol production plant using lignocellulosic substrate. Stress conditions included, apart from growth in the lignocellulosic substrate itself, elevated concentrations of glucose, NaCl, ethanol, and lactate as well as low pH. Results showed that, indeed, the strain adapted to lignocellulosic substrate also possessed the highest growth rate as well as shortest duration of the lag phase in this type of medium. However, the higher the additional stress level, the lower the difference compared to other strains, and X2180 in particular displayed a high resistance to these additional stress conditions. Furthermore, no difference in performance could be detected between the haploid or diploid versions of the laboratory strains. It might be that, at least under some circumstances, a laboratory strain such as X2180 could be an industrially attractive production organism with the advantage of facilitating the possibilities for making controlled genetic manipulations.

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Introduction

Biofuels and bioethanol are areas of research that are attracting a growing interest in order to reduce dependency on fossil fuels and to reduce net CO_2 emissions (see, e.g., [5, 12, 16]). Bioethanol formation using the yeast *Saccharomyces cerevisiae* and substrates such as sugar cane, wheat, and corn, is an established technique. However, there is a need to develop techniques that also include lignocellulosic substrates in order to reach sufficiently high volumes and to avoid competition with the food market. Unfortunately, however, lignocellulose as a substrate offers many challenges since it is rather nutrient-poor and even contains numerous growth-inhibiting substances [12, 14, 16]. Hence, the production organism needs to be well adapted to such conditions. It has turned out that *S. cerevisiae* is indeed an inhibitor-tolerant organism that can perform well with the proper cultivation procedures even in lignocellulosic media [3, 9, 11].

In general, it is believed that fermentation during harsh industrial conditions, especially with the additional constraints offered by lignocellulosic substrate, requires so-called industrial strains. These are characterized by having developed and become adapted over a long time in their specific environment, and genetically they are usually polyploid, aneuploid, or even alloploid [6, 15]. As a consequence, genetic manipulations, although feasible, are rather cumbersome. In contrast, the so-called academic or laboratory strains are often haploid and easily manipulated by using modern molecular biology tools. This fact makes such strains very attractive when there is a desire to change

their characteristics, for example, to produce novel compounds, increase the range of possible substrates and to use them simultaneously, and of course to increase formation rate and yield of any product. There are numerous examples of different ways to improve a biotechnological fermentation process. However, although attractive in this respect, laboratory strains in general are not expected to cope and perform well when brought into the real world of rather inhospitable industrial conditions.

To check whether this is always true, a comparison of growth and stress tolerance among industrial and laboratory *S. cerevisiae* strains was made in an industrial lignocellulosic substrate as well as in YPD. Two industrial strains were used, one of which was originally isolated from an industrial bioethanol production plant using lignocellulosic substrate, while the other one was a Swedish commercial baker's yeast strain. The laboratory strains included haploid and diploid versions of the commonly used CEN-PK and X2180 strains. Apart from the effect of the lignocellulosic substrate itself, additional stress factors of relevance for bioethanol production were explored. High gravity substrates are often used with concomitant high concentrations of particles and solutes. This makes the ability to manage osmotic stress an important feature [4], and hence the tolerance to elevated levels of glucose or NaCl in lignocellulosic media was examined. Similarly, the effect of elevated ethanol levels on the different strains was studied. Finally, a problematic fact with industrial ethanol production is that it is not performed under strict aseptic conditions and contamination with bacteria, mainly lactobacilli, is almost inevitably associated with the process [13]. A common practice to get rid of, or at least minimize, bacterial contamination is to introduce a very low pH at some point [4]. For this reason, investigations into the effect of low pH and the presence of a weak acid such as lactic acid under these conditions were performed.

Materials and methods

Yeast strains

The strains of *Saccharomyces cerevisiae* used included two industrial strains: a baker's yeast (Jästbolaget AB, Rotebro, Sweden, abbreviated JBA) and a strain used in ethanol production from lignocellulosic material (CCUG53310, Culture Collection University of Göteborg [11], abbreviated MoDo), and haploid and diploid versions of two laboratory strains: CEN.PK113-7D (mating type **a**) and CEN.PK122 (Euroscarf, Germany), and X2180-1A (mating type **a**) and X2180**a**α (Yeast Genetic Stock Center, Berkeley, CA, USA).

Media

Medium used were rich medium, YPD (10 g l^{-1} of yeast extract, 20 g l^{-1} of peptone, and 20 g l^{-1} of glucose), and an industrial medium, lignocellulosic hydrolysate. The lignocellulosic hydrolysate medium was composed of chips of spruce hydrolyzed with the dilute-acid method (SEKAB, Örnsköldsvik, Sweden), containing total carbohydrates (glucose equivalents) 292.4 mM, glucose 75.4 mM, mannose 52.7 mM, galactose 23.0 mM, fructose 0.0 mM, α-glucosides (glucose equivalents) 1.9 mM, β-glucosides (glucose equivalents) 137 mM, β-galactosides (galactose equivalents) 1.0 mM, acetic acid 47.4 mM, lactic acid 0.75 mM, phosphate 2.3 mM, and ammonia 0.43 mM (135 mM after pH adjustment, see below). Particles of the hydrolysate were removed either by filtration (Analytical Filter Papers, 1F, Munktell Filter, Grycksbo, Sweden) or by decantation. The pH of the hydrolysate was adjusted to 5.0 with ammonia and filter-sterilized. The pH-adjusted medium was used within 1 week after preparation.

The following additions/conditions were tested for both types of media: glucose 0–500 g l^{-1}, NaCl 0–100 g l^{-1}, ethanol 0–80 g l^{-1}, lactic acid 0–8 g l^{-1}, and pH in the range 3.0–6.0 (lignocellulose) or 3.0–7.0 (YPD). Another important parameter is temperature, which is especially relevant for SSF (see, e.g., [10, 18]) fermentations, but this will be the focus of a separate investigation.

Analyses of the hydrolysate

Glucose, galactose, acetate, and ammonium were determined by enzyme combination kits (R-Biopharm, Darmstadt, Germany), and fructose and mannose were determined enzymatically as described earlier [1]. Total carbohydrates were determined with the phenol method [7]. Glucosides were determined by treating diluted samples with 70 U ml^{-1} of α-glucosidase (from *Aspergillus niger*, Fluka Chemie, Buchs, Switzerland) in 25 mM Na–acetate buffer, pH 4.9, at 60°C overnight, or with 16.5 U ml^{-1} of β-glucosidase (from almonds, Fluka Chemie, Buchs, Switzerland) in 50 mM of Na–acetate buffer, pH 4.9, at 37°C overnight. The reaction was stopped by heating at 95°C. Samples were centrifuged for 5 min at 16,000×*g*, and liberated glucose was determined. α-Mannosides were determined by using 1 U ml^{-1} of α-mannosidase (from Jack Bean, Sigma–Aldrich Chemie, Steinheim, Germany) in 50 mM NaCitrate buffer, pH 4.5, at 25°C overnight, and β-galactosides were measured with the β-galactosidase of the galactose/lactose kit (R-Biopharm, Darmstadt, Germany) according to the instructions in the kit. Phosphate was measured colorimetrically at 25°C according to Bencini [2].

Table 1 Specific growth rate (μ) and length of lag phase during growth in rich YPD medium and lignocellulosic substrate for six different yeast strains[a]

Strain	μ (h^{-1})		Lag phase (h)	
	YPD	Lignocellulose	YPD	Lignocellulose
MoDo	0.55 ± 0.05	0.11 ± 0.04	3.3 ± 0.0	6.8 ± 1.6
JBA	0.57 ± 0.03	0.05 ± 0.01	4.0 ± 0.3	14.5 ± 1.3
CEN-PK haploid	0.50 ± 0.01	0.05 ± 0.00	3.0 ± 0.0	11.7 ± 0.4
CEN-PK diploid	0.49 ± 0.01	0.05 ± 0.00	3.2 ± 0.2	12.5 ± 0.3
X2180 haploid	0.48 ± 0.02	0.08 ± 0.00	3.2 ± 0.2	10.5 ± 0.2
X2180 diploid	0.52 ± 0.02	0.07 ± 0.02	3.0 ± 0.4	10.2 ± 0.2

[a] Each strain was cultivated at least in triplicate and the standard deviation (SD) is shown

Cultivations

Cultivations were performed at 30°C in 96-well plates with a culture volume of 350 µl as described by [17] using a computer-controlled incubator/reader/shaker (Bioscreen C, Thermic Labsystems, Oy, Finland). Pre-cultures were grown in YPD, and the inoculums were chosen to result in an initial OD_{610} of 0.03 and 0.05 for YPD and lignocellulose cultures, respectively. Growth was followed for 48 h, and triplicate cultures of each condition were done. To obtain OD values, the raw data of the bioscreen (BS) were recalculated to adjust for nonlinearity because of higher cell densities, according to $OD = (BS - 0.067) + 0.8324057 \times (BS - 0.067)^3$. The maximum specific growth rates were calculated by linear regression of natural logarithmic OD values from the exponential phase of the growth curves. The time of lag phase was defined as the time point where the OD was 1.5 times the starting OD.

The results presented are average values from at least three independent cultures, and the average SD for μ determinations were 9 and 10% (11 and 14% for the MoDo strain) during growth in YPD or lignocellulose, respectively. The corresponding average SD values for lag-phase determinations were 8% (11 and 20% for MoDo).

Results and discussion

Six different yeast strains were compared with respect to their growth performance and length of the lag phase under different stress conditions. Experiments were performed by using a rich YPD (yeast extract, peptone, glucose) medium and industrial lignocellulose-based substrate. Stress conditions included osmostress in the form of elevated levels of sugar (glucose) or salt (NaCl), ethanol tolerance, low pH, and presence of a weak acid such as lactic acid. The yeast strains tested were a haploid and diploid version of two commonly used laboratory strains, CEN-PK and X2180. The industrial strains were a Swedish commercial baker's yeast (JBA), and a strain that was previously isolated [11] from an industrial ethanol production plant based on lignocellulosic substrate (MoDo).

Growth rate and length of lag phase in YPD and lignocellulosic substrate

There were only very small differences in terms of growth rate as well as length of lag phase between the different strains during growth in YPD medium (Table 1). The length of the lag phase was 3–4 h in all cases, and the growth rate of the slowest growing strain was still more than 80% of the value obtained for the one with the highest growth rate, rendering all values statistically similar. A different situation emerged when using a lignocellulose-based medium. As expected, the MoDo strain, originally isolated from such an environment, was superior in coping with this more challenging substrate (Table 1). A two-tailed Student's *t*-test revealed that the reduction in growth rate and prolongation of lag phase was statistically($P < 0.05$) least severe for this strain compared to all other strains tested. The other industrial strain, JBA, did not, however, show any competitive advantage in the lignocellulosic environment over the laboratory strains. Similarly, no difference in performance could be detected between the haploid and diploid versions of the laboratory strains (Table 1).

Tolerance to high sugar concentrations in YPD and lignocellulosic substrate

The effect of high sugar concentrations on growth rate and length of the lag phase was very similar for all the strains when using YPD medium (Fig. 1a, b). There was a tendency towards a slightly longer lag phase for the MoDo strain at the highest glucose concentrations. However, it should be noted that even the highest glucose concentration of 500 g l^{-1} still supported a growth rate at or above 0.2 h^{-1} for all the strains.

As mentioned previously, the MoDo strain showed a superior performance compared to the other strains in the lignocellulosic medium. This competitive advantage was lost, however, when this medium was fortified with increasing concentrations of glucose (Fig. 1c). Already at a glucose concentration of 100 g l^{-1}, the haploid and diploid versions of the laboratory strain X2180 showed a similar performance to the MoDo strain. The other industrial strain JBA and laboratory CEN-PK strains that were less fit to

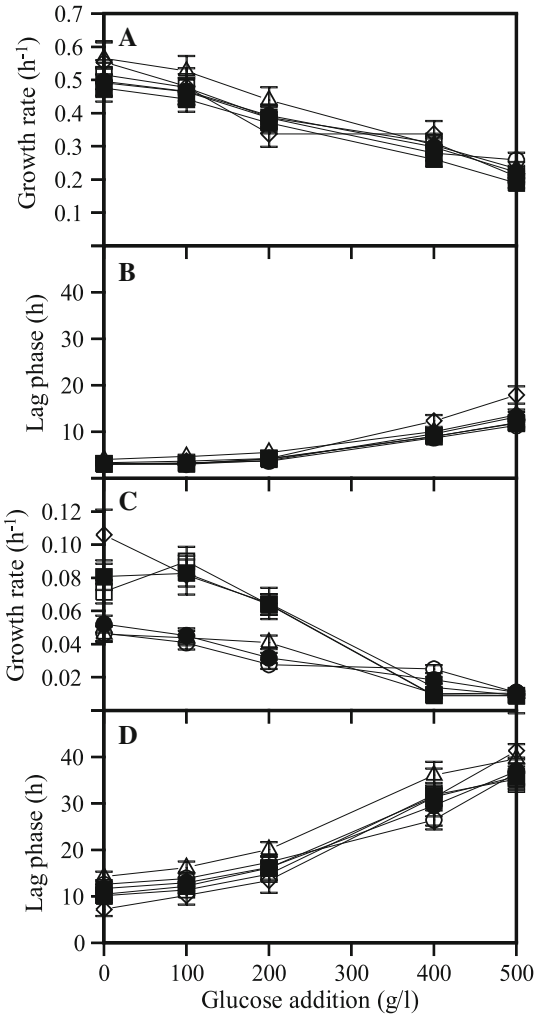

Fig. 1 Growth rate and length of lag phase during cultivation of different strains of *S. cerevisiae* at different glucose concentrations in YPD medium (**a**, **b**) or lignocellulosic medium (**c**, **d**). The strains tested were MoDo (*open diamond*), JBA (*open triangle*), X2180 haploid (*filled square*), X2180 diploid (*open square*), CEN-PK haploid (*filled circle*), and CEN-PK diploid (*open circle*). Each strain was cultivated in triplicate, and *error bars* show the mean standard deviation (SD)

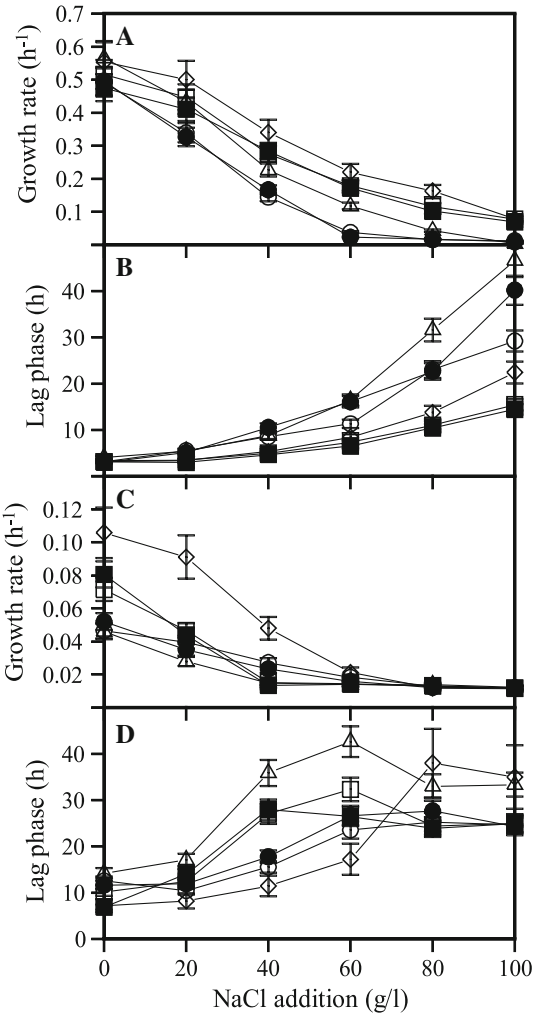

Fig. 2 Growth rate and length of lag phase during cultivation of different strains of *S. cerevisiae* at different NaCl concentrations in YPD medium (**a**, **b**) or lignocellulosic medium (**c**, **d**). The strains tested were MoDo (*open diamond*), JBA (*open triangle*), X2180 haploid (*filled square*), X2180 diploid (*open square*), CEN-PK haploid (*filled circle*), and CEN-PK diploid (*open circle*). Each strain was cultivated in triplicate, and *error bars* show the mean standard deviation (SD)

cope with the lignocellulosic substrate managed to maintain their relatively modest growth rates up to a glucose concentration of 200 g l^{-1} (Fig. 1c). At the highest glucose concentrations, the performance of all the strains was very similar, and very low growth rates were obtained, i.e., below 0.02 h^{-1} at 500 g l^{-1} of glucose. The length of the lag phase was of course prolonged at higher glucose concentrations in the lignocellulosic media also, but similar values were obtained for all strains tested (Fig. 1d).

Tolerance to high salt concentrations in YPD and lignocellulosic substrate

Increasing the osmotic pressure with NaCl showed inhibitory effects at much lower concentrations than glucose, similar to what has been reported previously [8]. Modo and X2180 strains were the ones most resistant to NaCl (Fig. 2a–d). In YPD medium, growth of these strains was supported at a rate close to 0.1 h^{-1} even at 100 g l^{-1} of NaCl (Fig. 2a). In contrast, JBA and the CEN-PK strains showed growth rates close to zero and/or very long lag phases at the highest NaCl concentrations (Fig. 2a, b).

In lignocellulosic medium, the MoDo strain maintained its competitive advantage compared to the other strains up to an NaCl concentration of 60 g l^{-1} with the highest growth rate as well as the shortest lag phase (Fig. 2c, d). Higher concentrations provoked a sharp increase in the length of the lag phase for the MoDo strain, and the growth rate fell below 0.02 h^{-1} for all strains tested. Similar to the case with glucose addition, no difference in stress tolerance

could be detected between the haploid and diploid versions of the laboratory strains X2180 and CEN-PK. Both of these strains and especially X2180 also displayed a higher resistance towards NaCl in lignocellulosic media compared to the industrial counterpart, JBA (Fig. 2c, d).

Tolerance to high ethanol concentrations in YPD and lignocellulosic substrate

There were no large differences among the strains at different ethanol concentrations when using YPD medium (Fig. 3a, b). The length of the lag phase was more or less identical, but the industrial strain, JBA, showed the highest growth rate throughout the entire range of ethanol concentrations tested. In fact, the growth rate at 8% (w/v) ethanol was as high as 0.3 h^{-1} for this strain.

The situation when using lignocellulosic media was completely different. In this environment, JBA had the lowest growth rate and among the longest lag phases at all ethanol concentrations tested (Fig. 3c, d). Instead, it was again MoDo that was the most resistant. Up to an ethanol concentration of 4%, this strain had the highest growth rate and the length of the lag phase was among the shortest. There seems to be something of a threshold value at 4–6% of ethanol for all strains tested as there was a drastic reduction in growth rate as well as prolongation of the lag phase in this interval (Fig. 3c, d). Similar to previous conditions, no difference between haploid and diploid strains could be detected, and X2180 was more resistant compared to CEN-PK.

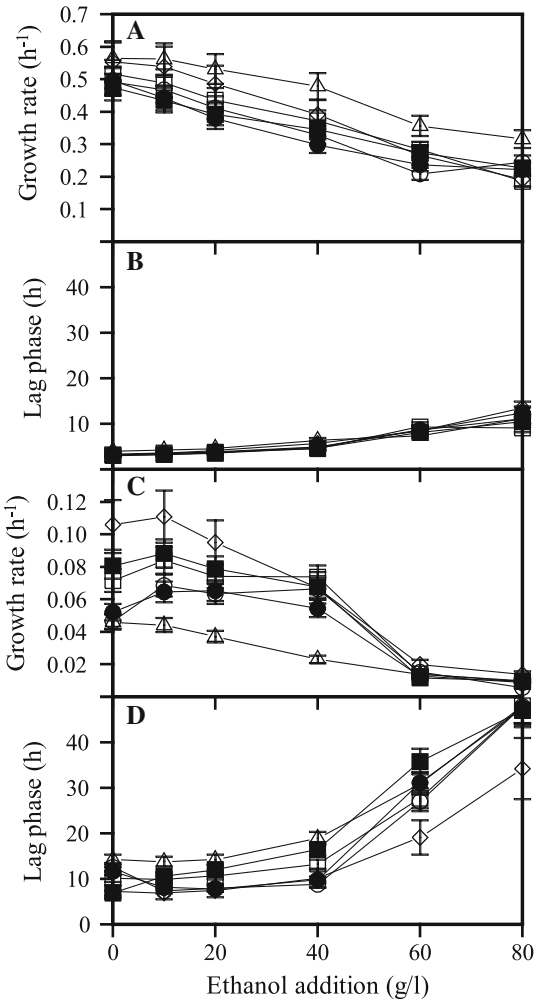

Fig. 3 Growth rate and length of lag phase during cultivation of different strains of *S. cerevisiae* at different ethanol concentrations in YPD medium (**a**, **b**) or lignocellulosic medium (**c**, **d**). The strains tested were MoDo (*open diamond*), JBA (*open triangle*), X2180 haploid (*filled square*), X2180 diploid (*open square*), CEN-PK haploid (*filled circle*), and CEN-PK diploid (*open circle*). Each strain was cultivated in triplicate, and *error bars* show the mean standard deviation (SD)

Tolerance to low pH in YPD and lignocellulosic substrate

No difference in growth performance could be detected between the strains at the various pH levels tested in YPD medium. There was a slight decrease in growth rate and increase in time for the lag phase when pH shifted from 4.0 to 3.0, but the response was similar for all strains (Fig. 4a, b).

As expected, the effect of low pH was more severe when using lignocellulosic media. This was manifested as a drastic reduction in growth rate and prolongation of the lag phase already at a change in pH from 5.0 to 4.0 (Fig. 4c, d). Again, the MoDo strain was the most resistant followed by X2180, and JBA and CEN-PK.

Tolerance to lactic acid in YPD and lignocellulosic substrate

The effect of lactic acid concentrations was tested in the range from 0 to 8 g l^{-1} in YPD as well as lignocellulosic media. Irrespective of substrate, no effect was detected in this range of concentrations for any of the strains tested (data not shown).

Conclusion

The strain originally isolated from an industrial ethanol production plant using lignocellulosic substrate, MoDo, did indeed show the best performance in this type of medium. However, the laboratory strains were also able to survive and multiply in this type of environment. In fact, the laboratory strain X2180 managed very well, and it clearly outcompeted the other industrial strain tested, JBA. Furthermore, the competitive advantage of Modo diminished when additional stress factors, such as osmotic, ethanol, low pH, or weak acid stress, were introduced on top of

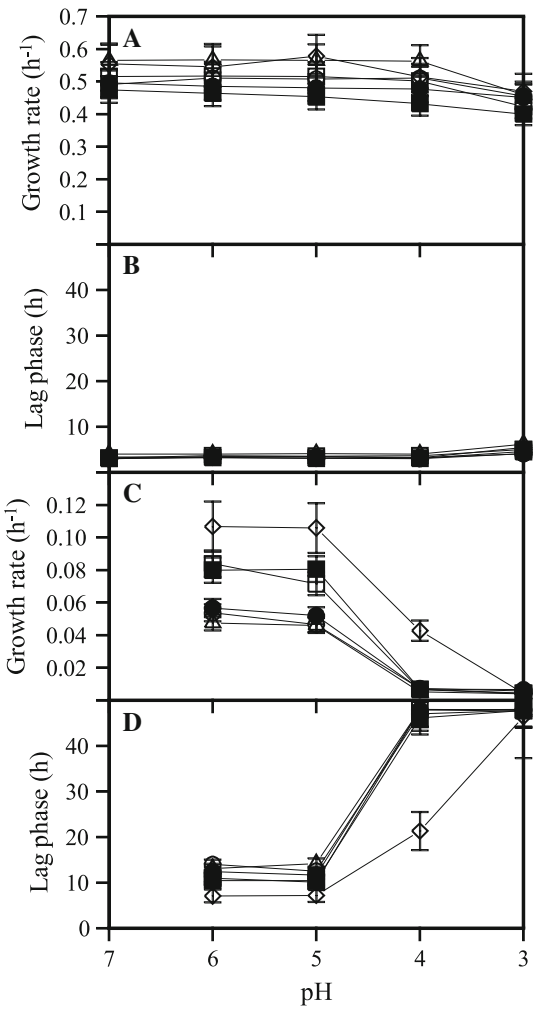

Fig. 4 Growth rate and length of lag phase during cultivation of different strains of *S. cerevisiae* at different external pH in YPD medium (**a**, **b**) or lignocellulosic medium (**c**, **d**). The strains tested were MoDo (*open diamond*), JBA (*open triangle*), X2180 haploid (*filled square*), X2180 diploid (*open square*), CEN-PK haploid (*filled circle*), and CEN-PK diploid (*open circle*). Each strain was cultivated in triplicate, and *error bars* show the mean standard deviation (SD)

the ones imposed by the lignocellulosic substrate itself, i.e., the greater the additional stress level, the lower the difference between the strains. A similar observation was reported by Martin and Jönsson [9] where one of the laboratory strains was found to be among the most resistant to a cocktail of lignocellulose-derived fermentation inhibitors.

It might be that a laboratory strain such as X2180 could be an attractive candidate as a production organism also for industrial scale ethanol production processes performed under harsh conditions. Not only would it very much simplify the procedure of making controlled specific genetic changes compared to the use of a traditional, usually polyploidy, industrial strain, but there is also a possibility that such a strain would respond much faster to variations in the environment and adapt itself accordingly in a continuous evolutionary engineering process.

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