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# Characterization of a Recombinant Flocculent *Saccharomyces cerevisiae* Strain That Co-Ferments Glucose and Xylose: II. Influence of pH and Acetic Acid on Ethanol Production

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**Abstract** The inhibitory effects of pH and acetic acid on the co-fermentation of glucose and xylose in complex medium by recombinant flocculent *Saccharomyces cerevisiae* MA-R4 were evaluated. In the absence of acetic acid, the fermentation performance of strain MA-R4 was similar between pH4.0–6.0, but was negatively affected at pH2.5. The addition of acetic acid to batch cultures resulted in negligible inhibition of several fermentation parameters at pH6.0, whereas the interactive inhibition of pH and acetic acid on the maximum cell and ethanol concentrations, and rates of sugar consumption and ethanol production were observed at pH levels below 5.4. The inhibitory effect of acetic acid was particularly marked for the consumption rate of xylose, as compared with that of glucose. With increasing initial acetic acid concentration, the ethanol yield slightly increased at pH5.4 and 6.0, but decreased at pH values lower than 4.7. Notably, ethanol production was nearly completely inhibited under low pH (4.0) and high acetic acid (150–200 mM) conditions. Together, these results indicate that the inhibitory effects of acetic acid and pH on ethanol fermentation by MA-R4 are highly synergistic, although the inhibition can be reduced by increasing the medium pH.

**Keywords** Recombinant *Saccharomyces cerevisiae* · Xylose · Glucose · Ethanol · Co-fermentation · pH · Acetic acid

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## Introduction

The increasing demand for renewable liquid transportation fuels has recently increased interest in lignocellulosic biomass as an abundant energy source for the industrial production of ethanol. However, the utilization of lignocellulosic biomass as an ethanol feedstock requires overcoming at least two technical hurdles during fermentation that are not faced with the use of starchy crops. Firstly, the yeast most commonly used for ethanol fermentation, *Saccharomyces cerevisiae*, cannot ferment xylose, a pentose sugar that composes a substantial fraction of lignocellulosic hydrolysates [1, 2]. Secondly, lignocellulosic hydrolysates contain various inhibitory compounds that negatively affect cell growth, ethanol yield, and fermentation productivity [3, 4]. Therefore, the development of robust *S. cerevisiae* strains that are able to tolerate inhibitors and efficiently ferment all sugars, including xylose, is required for ethanol production from lignocellulosic hydrolysates on an industrial scale.

The fermentation inhibitors typically found in lignocellulosic hydrolysates include weak acids, furan derivatives (e.g., furfural and hydroxymethylfurfural), and phenolic compounds. Acetic acid, which is present in all types of biomass at varying concentrations, is a weak acid inhibitor generated by the deacetylation of hemicellulose during the pretreatment of lignocellulosic biomass [3]. Inhibition by acetic acid is more severe in xylose-rich hydrolysates, such as those derived from hardwoods, which contain highly acetylated hemicelluloses and a high proportion of pentoses, than those from softwoods, which have a lower acetylated hemicellulose and pentose content [5]. Concentrations of acetic acid typically range from 1 to 10 g/L in lignocellulosic hydrolysates [6], although the level depends on the biomass source and pretreatment method. For example, acetic acid concentrations as high as 13 g/L have been observed in dilute acid-pretreated corn stover hydrolysate [7]. Therefore, detailed study of the influence of acetic acid on ethanol fermentation performance, particularly the utilization of xylose, is important for the engineering of microorganisms suitable for lignocellulosic ethanol commercialization.

pH is another important parameter that affects the inhibition of lignocellulose fermentation induced by weak acids, including acetic acid. Low pH inhibits cell proliferation and viability as a result of the increased proton gradient across the plasma membrane [8]. Thus, maintaining a neutral intracellular pH is crucial for cell viability. Although the optimal external pH range for the growth of *S. cerevisiae* is between 5.0 and 5.5 [9], cells can grow at pH levels as low as 2.5 in the absence of acetic acid [10]. However, the minimum pH at which *S. cerevisiae* cells can grow increases to 4.5 in the presence of acetic acid (10 g/L), with severely reduced growth rates being reported below pH4.5 [10]. To improve the overall efficiency of ethanol fermentation from lignocellulosic biomass and reduce production costs, it is thus necessary to remove acetic acid and adjust the external pH after pretreatment of the starting material. However, few inhibition studies on the synergistic effects of acetic acid and pH on engineered *S. cerevisiae* strains with xylose-utilizing ability have been reported.

The objectives of the present study were to assess not only the impact of medium pH but also the interactive impact of pH and acetic acid, on the co-fermentation of glucose and xylose to ethanol by the diploid industrial yeast strain *S. cerevisiae* MA-R4. Strain MA-R4 was previously genetically engineered for xylose metabolism by overexpressing xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Scheffersomyces* (*Pichia*) *stipitis*, and xylulokinase (XK) from *S. cerevisiae* in the xylulose-utilizing flocculent *S. cerevisiae* strain IR-2 [11]. Using strain MA-R4, we examined the effects of pH and acetic acid on several fermentation parameters, including cell growth, substrate utilization, ethanol concentration, and ethanol and by-product yields.

## Materials and Methods

### Microorganism and Media

The xylose-fermenting recombinant *S. cerevisiae* strain MA-R4, which was derived from the diploid and flocculent yeast strain IR-2 [12], was used in this study. MA-R4 was previously genetically engineered to express chromosomally integrated *XYL1* and *XYL2* genes, from *S. stipitis*, encoding XR and XDH, respectively, along with the endogenous *XKS1* gene encoding XK, under control of the *PGK* promoter [13]. For the construction of strain MA-R4, plasmid pAUR-XKXDHXR [14] was digested with the restriction enzyme *Bsi*WI and chromosomally integrated into the *aur1* locus of IR-2. MA-R4 was maintained on yeast peptone (YP) medium (10 g/L yeast extract and 20 g/L peptone) supplemented with 20 g/L glucose (YPD medium) and 0.5 mg/L aureobasidin A (Takara Bio, Kyoto, Japan). Glucose (45 g/L) and xylose (45 g/L) were added to YP medium to prepare YPDX medium, which was used as the fermentation medium in this study due to its extensive use in previous studies examining the fermentation performance of MA-R4 [13, 15]. Glucose and xylose were selected as representative hexose and pentose monosaccharides, respectively, as they are both major components of lignocellulosic hydrolysates.

The pH of the YPDX medium was adjusted to 2.5, 4.0, and 5.5 by the addition of 8 M KOH. For experiments involving acetic acid, YPDX medium was supplemented with acetic acid prior to pH adjustment at final concentrations of 0 (control), 50, 100, 150, and 200 mM. The pH of the medium was then adjusted to 6.0, 5.4, 4.7, and 4.0 with 8 M KOH.

### Fermentation

For anaerobic batch fermentation, MA-R4 was first cultivated aerobically in 5 mL YPD medium for 36 h at 30 °C. The resulting culture was centrifuged at 6,000×*g* for 5 min at 4 °C, and the pelleted cells were then washed and resuspended in 1 mL distilled water. The washed cells were inoculated into 20 mL fermentation medium (YPDX with or without acetic acid) to give an initial cell density of approximately 4.01 g (dry cell weight (DCW)) per liter. Anaerobic batch fermentations were performed at 30 °C in 50-mL sterilized closed bottles with magnetic stirring, as described previously [13, 14]. Samples (0.3 mL) of fermentation broth were collected at specified intervals and diluted 4-fold with 8 mM $H_2SO_4$. The diluted samples were stored at −30 °C for high-performance liquid chromatography (HPLC) analysis of substrates and fermentation products. All experiments were repeated three times.

### Analytical Methods

DCW was determined by measuring the absorbance of diluted culture samples at 600 nm using a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan), as described previously [14]. Fermentation metabolites were analyzed with an HPLC apparatus (Jasco, Tokyo, Japan) equipped with a refractive index detector (RI-2031Plus; Jasco) using Aminex HPX-87 H (Bio-Rad Laboratories, Hercules, CA, USA) and Cation H Refill Guard (Bio-Rad) columns. HPLC was performed at 65 °C using 5 mM $H_2SO_4$ as the mobile phase, a flow rate of 0.6 mL/min, and an injection volume of 20 μL.

## Results and Discussion

### Effect of pH on the Co-Fermentation of Glucose and Xylose

As the lignocellulosic starting material used for ethanol production is typically acidic due to pretreatment with diluted acid, we investigated the effects of acidic pH (5.5, 4.0, and 2.5) on the co-fermentation of glucose and xylose by the XR/XDH/XK-expressing recombinant *S. cerevisiae* strain MA-R4 (Fig. 1). At all three pH levels, MA-R4 displayed simultaneous co-

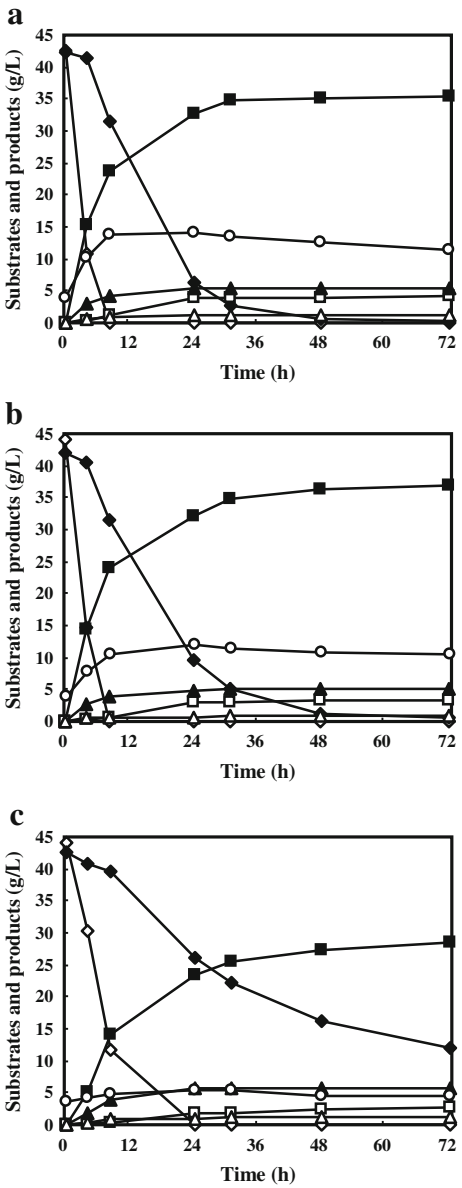


**Fig. 1** Time-dependent ethanol fermentation profiles of recombinant *S. cerevisiae* strain MA-R4 at pH **a** 5.5, **b** 4.0, and **c** 2.5. *white diamond*, glucose; *black diamond*, xylose; *black square*, ethanol; *white square*, xylitol; *black triangle*, glycerol; *white square*, acetic acid; *white circle*, dry cell weight. Values are averages from three independent experiments

fermentation of glucose and xylose, although preferentially utilized glucose (Fig. 1a, 0 to 8 h, pH5.5; Fig. 1b, 0 to 8 h, pH4.0; Fig. 1c, 0 to 24 h, pH2.5), as was observed in an accompanying paper [11]. In the fermentations conducted at pH levels 5.5 and 4.0, MA-R4 exhibited rapid growth during the glucose and xylose co-consumption phase (0 to 8 h; Fig. 1a, b), but grew slowly or poorly in the xylose-only consumption phase (8 to 72 h; Fig. 1a, b). At pH2.5, MA-R4 displayed poor growth during mixed-sugar fermentation (Fig. 1c), a finding that is consistent with a previous report demonstrating that the growth of *S. cerevisiae* is inhibited at lower pH [9]. The maximum cell concentration after 72 h of fermentation at pH levels 5.5, 4.0, and 2.5 was 14.07, 12.13, and 5.41 g/L, respectively. When cultured at pH levels 5.5 and 4.0, MA-R4 consumed glucose within the initial 8 h of fermentation, while xylose was almost completely consumed within 48 h (Fig. 1a, b). In contrast, MA-R4 fermented glucose and xylose much more slowly at pH2.5, under which conditions glucose was consumed within 24 h and only approximately 72 % of the xylose was consumed after 72 h (Fig. 1c).

At the end of the 72-h fermentation, the highest ethanol concentration produced by MA-R4 at pH levels 5.5, 4.0, and 2.5 was 35.35 g/L (Fig. 1a), 36.79 g/L (Fig. 1b), and 28.39 g/L (Fig. 1c), respectively. The highest consumption rates of glucose and xylose (7.915 and 1.662 g/L h, respectively) were observed at pH5.5. However, the maximum ethanol production rate (2.988 g/L h) of MA-R4 during fermentation at pH4.0 was nearly identical to that (2.979 g/L h) at pH5.5. The ethanol yield per gram of total consumed sugars (gram per gram) was also similar (0.42–0.43 g/g) for MA-R4 cells cultured at pH levels 5.5 and 4.0, with the ethanol yield (0.38 g/g) clearly decreasing at pH2.5. Based on these results, the pH range of 5.5 to 4.0 was considered to allow for the most efficient ethanol fermentation of glucose and xylose by MA-R4.

## Combined Effect of pH and Acetic Acid on the Co-Fermentation of Glucose and Xylose

To examine the combined effects of acetic acid concentration and medium pH on the fermentation performance of MA-R4, five concentrations of acetic acid (0, 50, 100, 150, and 200 mM) and four medium pH level (4.0, 4.7, 5.4, and 6.0) were evaluated in several combinations to give a total of 20 fermentation conditions. The glucose, xylose, and ethanol concentration profiles during glucose and xylose co-fermentation for the various conditions were then measured (Fig. 2). Several fermentation parameters were also examined and are shown in Table 1.

In the absence of added acetic acid, a reduction of the external pH from 6.0 to 4.0 had only a limited impact on the fermentation performance of MR-R4, as indicated by the concentrations of glucose, xylose, and ethanol (Fig. 2) and several of the fermentation parameters (Table 1). At all examined pH levels, MA-R4 fermented glucose and xylose within 8 and 48 h, respectively (Fig. 2, upper panels). Based on these results, MA-R4 appeared to rapidly produce ethanol during the co-fermentation of glucose and xylose (0–8 h), and then produce ethanol more gradually from xylose after the consumption of glucose (Fig. 2; 8–72 h, lower panels). In addition, the maximum production rates, concentrations, and yields of ethanol for each pH condition were relatively constant during fermentation in the absence of exogenously added acetic acid (Table 1). These results are consistent with our finding that MA-R4 exhibited similar fermentation patterns at pH levels 5.5 and 4.0 (Fig. 1). However, we observed the following minor differences in the fermentation activity of MR-R4 at different pH levels when acetic acid was not included the medium: (1) the maximum cell concentration and glucose consumption rate were slightly higher at pH5.4 compared to those at other pH values; (2) the maximum rate of xylose consumption was slightly lower at

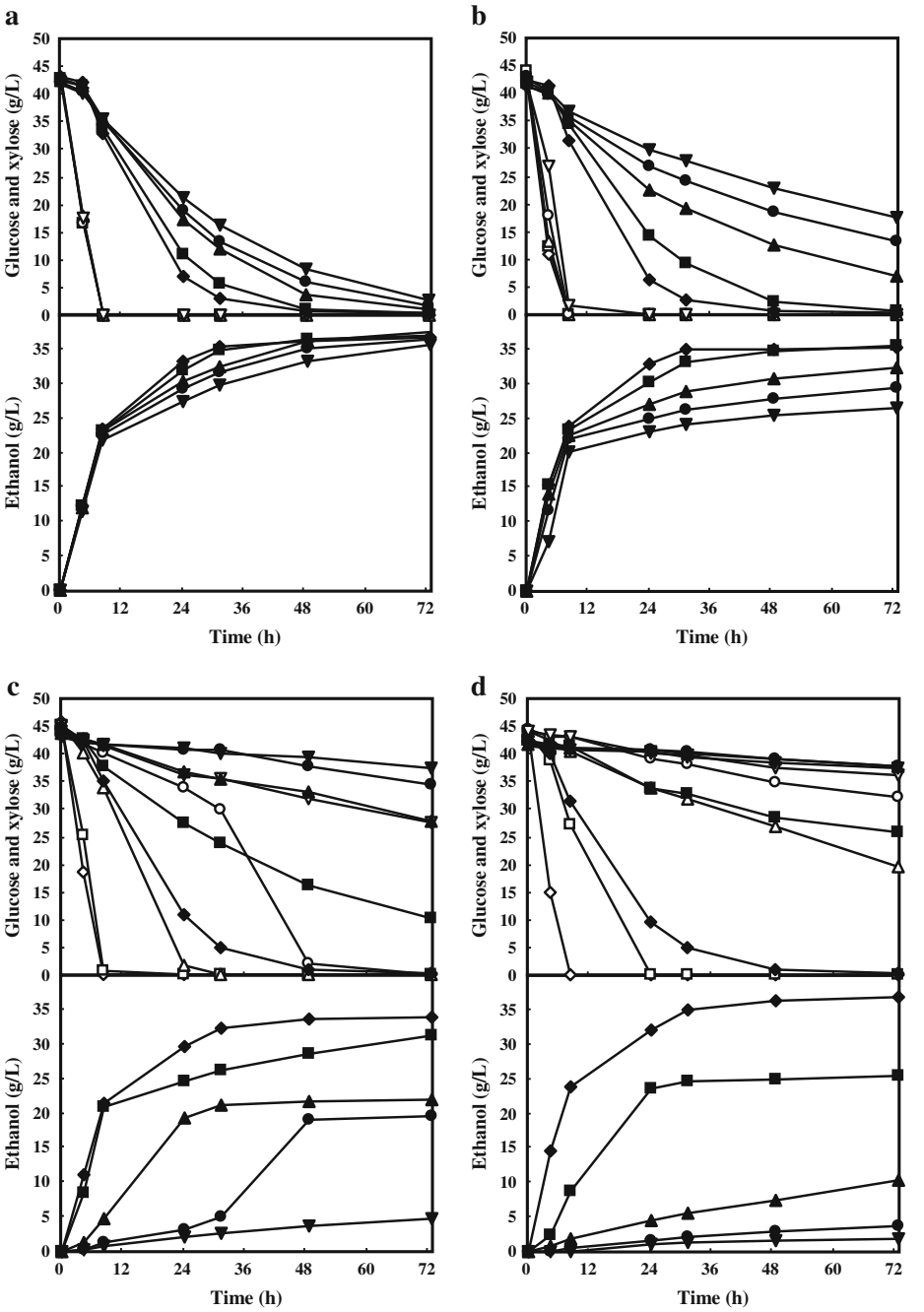


**Fig. 2** Time-dependent ethanol fermentation profiles of glucose (upper panels, open symbols) and xylose (upper panels, closed symbols), and ethanol concentrations (lower panels) by recombinant *S. cerevisiae* strain MA-R4 in the presence of varying initial concentrations of acetic acid at pH values of **a** 6.0, **b** 5.4, **c** 4.7, and **c** 4.0. Acetic acid was added to the fermentation medium at final concentrations of 0 mM (*black diamond*), 50 mM (*black square*), 100 mM (*black up-pointing triangle*), 150 mM (*black circle*), or 200 mM (*black down-pointing triangle*). Values are averages from three independent experiments

**Table 1** Fermentation results at 72 h for *S. cerevisiae* strain MA-R4 cultured at different acetic acid concentrations and pH values in medium containing 45 g/L glucose and xylose

| pH | Acetic acid (mM) | Max. cell concentration (g/L) | Max. ethanol concentration (g/L) | Max. glucose consumption rate (g/L·h) | Max. xylose consumption rate (g/L·h) | Max. ethanol production rate (g/L·h) | Ethanol yield (g/g) | Xylitol yield (g/g) | Glycerol yield (g/g) |
|---|---|---|---|---|---|---|---|---|---|
| 6.0 | 0 | 11.67±0.36 | 36.5±0.32 | 6.376±0.307 | 1.746±0.096 | 2.926±0.017 | 0.423±0.018 | 0.107±0.002 | 0.075±0.000 |
| | 50 | 12.09±0.39 | 36.8±0.50 | 6.448±0.060 | 1.512±0.060 | 2.869±0.020 | 0.430±0.008 | 0.090±0.003 | 0.065±0.002 |
| | 100 | 10.66±0.92 | 37.2±0.25 | 6.274±0.335 | 1.207±0.130 | 2.884±0.022 | 0.439±0.018 | 0.074±0.002 | 0.066±0.002 |
| | 150 | 11.46±0.53 | 36.3±0.74 | 6.488±0.145 | 1.091±0.043 | 2.827±0.016 | 0.441±0.015 | 0.069±0.001 | 0.067±0.002 |
| | 200 | 9.90±0.74 | 35.4±0.71 | 6.092±0.208 | 0.953±0.009 | 2.730±0.032 | 0.442±0.008 | 0.060±0.003 | 0.065±0.000 |
| 5.4 | 0 | 15.00±0.46 | 35.4±0.69 | 7.915±0.373 | 1.762±0.024 | 2.979±0.037 | 0.423±0.015 | 0.103±0.003 | 0.067±0.003 |
| | 50 | 12.57±0.06 | 35.5±0.56 | 7.988±0.177 | 1.307±0.040 | 2.909±0.045 | 0.428±0.007 | 0.080±0.003 | 0.050±0.002 |
| | 100 | 10.57±0.50 | 32.4±1.74 | 7.491±0.287 | 0.884±0.030 | 2.812±0.073 | 0.432±0.004 | 0.076±0.007 | 0.049±0.003 |
| | 150 | 9.17±0.74 | 29.4±1.08 | 6.243±0.429 | 0.638±0.015 | 2.735±0.042 | 0.435±0.017 | 0.071±0.002 | 0.049±0.002 |
| | 200 | 7.10±0.17 | 26.4±0.49 | 5.028±0.350 | 0.533±0.011 | 2.521±0.132 | 0.437±0.013 | 0.059±0.015 | 0.051±0.004 |
| 4.7 | 0 | 12.51±0.60 | 34.0±0.94 | 6.817±0.441 | 1.561±0.059 | 2.689±0.121 | 0.424±0.001 | 0.085±0.009 | 0.054±0.002 |
| | 50 | 10.15±2.25 | 31.3±2.34 | 5.540±0.231 | 0.598±0.046 | 2.612±0.185 | 0.426±0.004 | 0.077±0.011 | 0.041±0.003 |
| | 100 | 7.53±0.90 | 22.0±1.06 | 1.822±0.136 | 0.224±0.086 | 0.808±0.092 | 0.391±0.007 | 0.071±0.013 | 0.041±0.004 |
| | 150 | 5.77±0.54 | 19.6±0.66 | 0.886±0.034 | 0.122±0.006 | 0.396±0.014 | 0.379±0.008 | 0.076±0.002 | 0.038±0.005 |
| | 200 | 4.11±0.75 | 4.74±2.15 | 0.241±0.063 | 0.076±0.006 | 0.068±0.028 | 0.207±0.042 | ND | 0.021±0.004 |
| 4.0 | 0 | 12.17±0.63 | 36.8±1.06 | 7.291±0.535 | 1.531±0.076 | 2.988±0.031 | 0.431±0.011 | 0.078±0.002 | 0.060±0.004 |
| | 50 | 7.42±0.64 | 25.5±0.22 | 2.942±0.512 | 0.465±0.017 | 1.577±0.126 | 0.427±0.021 | 0.106±0.008 | 0.045±0.005 |
| | 100 | 4.71±0.10 | 10.4±0.14 | 0.434±0.033 | 0.053±0.005 | 0.178±0.007 | 0.369±0.003 | ND | 0.022±0.003 |
| | 150 | 4.49±0.20 | 3.74±0.19 | 0.196±0.034 | 0.054±0.003 | 0.077±0.016 | 0.228±0.002 | ND | 0.020±0.001 |
| | 200 | 4.26±0.28 | 1.81±0.53 | 0.135±0.025 | 0.055±0.002 | 0.057±0.011 | 0.152±0.033 | ND | 0.025±0.001 |

Values are the averages of three independent experiments±standard deviation

*ND* not detectable

pH levels 4.7 and 4.0 compared with that at pH levels 6.0 and 5.4; and (3) the xylitol yield was reduced by decreasing the medium pH from 6.0 to 4.0 (Table 1). Taken together, our findings indicate that in the absence of added acetic acid, pH5.4–5.5 is the optimal pH for the co-fermentation of glucose and xylose by MA-R4. The results of more detailed analysis of the effects of acetic acid addition for each pH condition are described in the following sections.

## Combined Effect of pH and Acetic Acid on Cell Growth

When the initial medium pH was 6.0, the maximum cell concentration of MA-R4 remained relatively unchanged in spite of increasing concentrations of acetic acid, with the exception of medium supplemented with 200 mM acetic acid (Table 1). In contrast, at pH values lower than 5.4, the maximum cell concentration decreased as the concentration of acetic acid was increased (Table 1). Under the most severe fermentation conditions (pH4.0 and 100–200 mM acetic acid), the maximum cell concentration was only slightly higher than the initial concentration (Table 1). A general trend of reducing maximum cell concentration was observed with decreasing fermentation pH for each acetic acid concentration (Table 1). These results indicate that the pH-dependent inhibition of cell growth by the addition of acetic acid to the fermentation medium is more pronounced under low pH conditions. The observed decrease in cell concentration can be explained by the membrane transportability of acetic acid, as described in the classical weak-acid theory. Under these conditions, undissociated, uncharged molecules of acetic acid freely diffuse across the cytoplasmic membrane and dissociate in the cytoplasm owing to higher intracellular pH levels, leading to an inhibitory effect on normal cellular metabolism [16]. To maintain intracellular pH homeostasis, plasma membrane ATPase pumps the excess protons out of cell at the expense of ATP to avoid cytoplasmic acidification. The requirement for ATP causes a decrease in the total cellular biomass produced, which was detected here as reduced cell concentrations.

## Combined Effect of pH and Acetic Acid on Sugar Consumption

At pH6.0, MA-R4 completely consumed the glucose in the YPDX medium within the initial 8 h of fermentation and was able to metabolize nearly all of the xylose within 72 h, even at the highest concentrations of acetic acid (Fig. 2a, upper panel). A similar result was obtained for glucose consumption at fermentations conducted at pH5.4 (Fig. 2b, upper panel), whereas the amount of consumed glucose after 72 h of fermentation decreased with increasing acetic acid concentrations at pH values 4.7 and 4.0 (Fig. 2c, d; upper panels). Xylose consumption also decreased during the 72-fermentation period as the concentration of acetic acid increased at pH values between 5.4 and 4.0 (Fig. 2b–d, upper panels). MA-R4 was able to consume 98, 83, 68, and 57 % of the xylose with 72 h at acetic acid concentrations of 50, 100, 150, and 200 mM, respectively, for fermentations conducted at pH5.4 (Fig. 2b, upper panel). Notably, at pH4.7 and 200 mM acetic acid, and pH4.0 and 100–200 mM acetic acid, MA-R4 was unable to metabolize glucose or xylose within 72 h (Fig. 2c, d; upper panels). Under the most severe condition (pH4.0 and 200 mM acetic acid), only 18 and 10 % of the total glucose and xylose, respectively, was fermented by MA-R4 (Fig. 2d, upper panel). Thus, the consumption of sugars (glucose and xylose) by MA-R4 decreases with increasing initial acetic acid concentrations and xylose fermentation appears to be more sensitive than glucose fermentation to the presence of acetic acid at low pH.

The maximum glucose consumption rates for each pH condition decreased with increasing acetic acid concentration, with the exception of pH6.0, at which glucose consumption

remained nearly unaffected, even in the presence of 200 mM acetic acid (Table 1). Furthermore, the inhibitory effect of acetic acid on the glucose consumption rate became more pronounced as the acidity of the medium increased (Table 1). Under the harshest fermentation condition (pH4.0 and 200 mM acetic acid), no significant glucose consumption was observed. A decrease in the maximum xylose consumption rate was also observed with increasing acetic acid concentrations and decreasing pH (Table 1). Under the lowest pH condition (pH4.0), the addition of acetic acid at concentrations greater than 100 mM resulted in xylose consumption rates that were only approximately 8 % of the control fermentation (Table 1). These results strongly suggest that the xylose consumption rate of MA-R4 is more severely affected by the presence of acetic acid and low pH than the glucose consumption rate. The specific effect of acetic acid and/or pH on the fermentation of xylose has also been observed for other xylose-utilizing *S. cerevisiae* strains [17–19], suggesting that the ATP generation rate during xylose fermentation is lower than that during glucose fermentation [18].

## Combined Effect of pH and Acetic Acid on Ethanol Production

The amount of ethanol produced by MA-R4 during 72 h of fermentation at pH6.0 was similar (35.4–37.2 g/L; Fig. 2a, lower panel and Table 1) at all examined acetic acid concentrations because the growth and sugar consumption of MA-R4 was not inhibited by acetic acid at this higher pH value. In contrast, ethanol production decreased with increasing initial acetic acid concentrations at the other pH levels (pH5.4–4.0; Fig. 2b–d, lower panels), exhibiting a trend similar to that for glucose and xylose consumption. In fact, the maximum ethanol concentration decreased as the concentration of acetic acid increased in the pH range from 5.4 to 4.0 (Table 1). A similar trend was also detected for the maximum ethanol production rate; the rate decreased as the concentration of acetic acid in the medium increased (Table 1). In particular, significant decreases in the maximum concentrations and production rate of ethanol were observed at pH levels 4.7 and 4.0 in the presence of acetic acid (Table 1), suggesting the importance of controlling the pH of hydrolysates for improving ethanol fermentation performance. At the lowest pH, a 98 % reduction in the maximum ethanol production rate was observed when the concentration of acetic acid was raised from 0 to 200 mM.

Under pH conditions of 6.0 and 5.4, a slight increase in the yield of ethanol was seen with increasing acetic acid concentrations (Table 1), a finding that is in good agreement with the results of a study performed by Casey et al. [18] using the well-known xylose-fermenting *S. cerevisiae* strain 424A (LNH-ST). The increase in the ethanol yield can be explained by the weak acid theory, as described above. Specifically, in order for cells to meet the necessary ATP demand for maintenance and growth, a greater proportion of available sugar is converted to ethanol, which results in increased ethanol yield at the expense of biomass production [20]. However, the presence of acetic acid reduced the consumption of sugars and yield of ethanol under low pH conditions (pH levels 4.7 and 4.0; Table 1). In the presence of 200 mM acetic acid, ethanol yield decreases of 51 and 65 % were observed at pH levels 4.7 and 4.0, respectively, as compared with the respective controls (Table 1). Thus, an increase in the concentration of acetic acid inhibited the maximum concentration, production rate, and yield of ethanol by MA-R4 during the co-fermentation of glucose and xylose, particularly at low medium pH.

## Combined Effect of pH and Acetic Acid on Fermentation By-Products

Under all examined fermentation conditions, glycerol accumulation in the medium (≤6 g/L) was mainly observed during the glucose consumption phase. The concentrations of glycerol

decreased with increasing acetic acid concentrations for each pH, as well as with decreasing pH at a fixed acetic acid concentration. A similar trend was also observed for xylitol accumulation (≤4 g/L), although xylitol was not secreted by MA-R4 under the most severe conditions (pH4.7 and 200 mM acetic acid, pH4.0 and 100–200 mM acetic acid). The addition of electron acceptors such as acetoin, acetaldehyde, and furfural, has been shown to decrease xylitol accumulation by recombinant *S. cerevisiae* [21], and acetic acid has also been identified as an electron acceptor for mannitol fermentation [22]. Therefore, the most likely reason for reduced xylitol production observed at increased acetic acid levels is that MA-R4 was able to use acetic acid as an electron acceptor for co-fermentation of glucose and xylose. Interestingly, the xylitol yield decreased with increasing initial acetic acid concentrations at pH values 6.0 and 5.4 (Table 1), whereas the glycerol yield decreased with increasing initial acetic acid concentrations predominantly at pH values lower than 4.7 (Table 1). This finding is likely due to the fact that glycerol is formed by cells to reoxidize the NADH that originates during the synthesis of cellular material [10].

## Conclusions

The effects of different pH conditions in the presence and absence of acetic acid (0–200 mM) on the ethanol fermentation performance of the recombinant industrial *S. cerevisiae* strain MA-R4 were examined. In the absence of acetic acid (initial pH range of 6.0–4.0), the production rate and yield of ethanol by MA-R4 were not inhibited during the fermentation, whereas all examined fermentation parameters, including cell concentration, were negatively affected under strong acidic conditions (pH2.5), demonstrating the importance of maintaining the fermentation pH above 4.0 in the absence of acetic acid. At pH6.0, the addition of acetic acid led to negligible inhibition of a few fermentation parameters, including the rates of glucose consumption and ethanol production, although the xylose consumption rate correspondingly decreased. Thus, the consumption rate of xylose by MA-R4 is more remarkably affected than that of glucose in the presence of acetic acid. At the lower pH range of 5.4 to 4.0, initial acetic acid concentrations of higher than 50 mM negatively affected several fermentation parameters, particularly when coupled with low pH, confirming that the undissociated form of acetic acid acts as an inhibitor. Based on our present results, strictly controlling the acetic acid level and pH of the fermentation medium is important for the efficient co-fermentation of glucose/xylose by MA-R4 in the lignocellulosic ethanol production process. Therefore, it is necessary to improve the tolerance of MA-R4 against weak acids, particularly acetic acid, which are present in lignocellulosic hydrolysates, and to establish a pretreatment method that includes detoxification. The findings from our study further suggest that strictly controlling fermentation pH conditions may be a suitable remedial approach for minimizing the inhibitory effects of acetic acid. In the near future, we are planning to perform such work on real lignocellulosic hydrolysates instead of YPDX medium that usually tends to protect the yeast under stressful conditions.

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