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SHORT COMMUNICATION

# Increased ethanol production by deletion of *HAP4* in recombinant xylose-assimilating *Saccharomyces cerevisiae*

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**Abstract** The *Saccharomyces cerevisiae HAP4* gene encodes a transcription activator that plays a key role in controlling the expression of genes involved in mitochondrial respiration and reductive pathways. This work examines the effect of knockout of the *HAP4* gene on aerobic ethanol production in a xylose-utilizing *S. cerevisiae* strain. A *hap4*-deleted recombinant yeast strain (B42-DHAP4) showed increased maximum concentration, production rate, and yield of ethanol compared with the reference strain MA-B42, irrespective of cultivation medium (glucose, xylose, or glucose/xylose mixtures). Notably, B42-DHAP4 was capable of producing ethanol from xylose as the sole carbon source under aerobic conditions, whereas no ethanol was produced by MA-B42. Moreover, the rate of ethanol production and ethanol yield (0.44 g/g) from the detoxified hydrolysate of wood chips was markedly improved in B42-DHAP4 compared to MA-B42. Thus, the results of this study support the view that deleting *HAP4* in xylose-utilizing *S. cerevisiae* strains represents a useful strategy in ethanol production processes.

**Keywords** *Saccharomyces cerevisiae* · *HAP4* gene · Xylose · Ethanol production · Lignocellulosic hydrolysate

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## Introduction

Lignocellulosic biomass contains multiple fermentable sugars and represents a potentially valuable feedstock for fuels and chemicals. Bioethanol is the most dominant biofuel employed in the transportation sector at present. The economical conversion of lignocellulosic biomass into ethanol has gained increased attention due to the lack of competition with food resources as well as reduced greenhouse gas (GHG) emissions [1]. The pentose sugar xylose, which is the second-most abundant monosaccharide in nature following glucose, is present in significant amounts in lignocellulosic biomass hydrolysates. However, this sugar cannot be utilized by wild-type *Saccharomyces cerevisiae*. Although this species shows excellent ability to ferment hexose sugars such as glucose and high tolerance to ethanol and inhibitors found in lignocellulosic hydrolysates, baker's yeast lacks a catabolic pathway for the utilization of pentoses and so is unable to ferment xylose [2].

To date, genetic engineering approaches have enabled *S. cerevisiae* to utilize xylose by the expression of heterologous genes. Specifically, strains have been constructed that produce xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Scheffersomyces stipitis* or express a fungal or bacterial gene encoding xylose isomerase (XI); these gene products generate substrates for use by the endogenous xylulokinase (XK), the product of the *XKS1* gene [3, 4]. Further targeted metabolic engineering and evolutionary engineering approaches have been successfully employed to improve xylose fermentation (e.g., reviewed by [3, 4]). Efficient production of ethanol from xylose (let alone lignocellulosic hydrolysates) comparable to that from glucose will require further strain engineering.

Investigations using genome-wide transcription analysis have suggested that xylose-utilizing *S. cerevisiae* strains recognize xylose as a non-fermentable carbon source [5, 6]. Another study found that xylose also acts as a weakly repressive carbon source, less strongly than glucose [7, 8]. In addition, transcript levels of *HAP4*, which encode a transcriptional activator and global regulator of respiratory gene expression [9], were induced manyfold in the presence of xylose compared to induction in the presence of glucose [5, 6]. Thus, *S. cerevisiae* engineered for xylose metabolism does not exhibit a fermentative response to xylose even under anaerobic conditions, and the regulation of xylose metabolism in recombinant *S. cerevisiae* is similar to glucose metabolism in Crabtree-negative yeast [10]. Since the control of oxygen levels in cultures is complex and uneconomical for industrial ethanol production, it is preferable to develop respiration-independent *S. cerevisiae* strains for industrial ethanol production from xylose. Completely respiration-deficient nuclear petites may facilitate such applications [11]. Indeed, respiration-deficient mutants of *S. cerevisiae* strains displayed 30–43 % higher ethanol productivity than the respiratory-sufficient parent strain when glucose was used as a substrate [11, 12]. The use of respiration-deficient *S. stipitis* and *S. cerevisiae* strains has been shown to improve ethanol yield [13, 14]. Recently, Peng et al. [15] reported the adaptive evolution of a respiration-deficient *S. cerevisiae* strain harboring multiple genetic modifications, permitting major improvements in anaerobic growth, xylose consumption, and ethanol yield. However, few reports are available on production of ethanol by respiration-deficient *S. cerevisiae* strains with xylose-assimilating ability, including the application of such strains in the production of ethanol from lignocellulosic hydrolysates.

Earlier work demonstrated increased ethanol production in *S. cerevisiae hap4* mutants grown in microaerated chemostats with glucose as the sole carbon source [16]. Further elevation of ethanol yield was reported in a *hap4*Δ mutant of *Pichia guilliermondii* during aerobic fermentation using glucose [17]. Interestingly, Lin et al. [18] showed that deletion of *HAP4* improves cellobiose fermentation. These results suggested that ethanol production could also be improved by manipulating the regulators of respiration. In contrast, *S. cerevisiae* overexpressing *HAP4* exhibited decreased ethanol production and increased biomass production when grown on glucose in aerobic culture [19]. In combination with the above-mentioned work, these results suggested that deletion of *HAP4* in a xylose-utilizing *S. cerevisiae* would permit increased production of ethanol from both xylose and mixtures of glucose and xylose, even under aerobic conditions. Therefore, the aim of this study was to examine the impacts of *hap4* deletion on ethanol production by a xylose-utilizing *S. cerevisiae* strain. Several parameters were evaluated for aerobic cultures growing in complex medium containing glucose and/or xylose and in a lignocellulosic Japanese cedar-based hydrolysate.

## Materials and methods

### Yeast strains and media

The wild-type *S. cerevisiae* strain, BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*), and the knockout strain, *hap4*Δ in a BY4742 strain background (Open Biosystems, Huntsville, AL, USA), were used in this study. The plasmid pAUR-XKXDHXR [20], which was used for XK, XDH, and XR expression driven by the yeast phosphoglycerate kinase (*PGK*) promoter, was digested with *Bsi*WI and chromosomally integrated into the *aur1* locus of BY4742 and that of the *hap4*Δ mutant to construct the recombinant strains MA-B42 and B42-DHAP4, respectively. The respiratory-deficient strain B42-DHAP4 and control strain MA-B42 were grown in yeast peptone (YP) medium (10 g/L yeast extract and 20 g/L peptone) supplemented with 20 g/L glucose (YPD medium). Aureobasidin A (Takara Bio, Kyoto, Japan) was added at 0.5 mg/L to the above-described medium for maintenance of selective growth. For aerobic batch cultivation, a mineral-based (M) medium containing 7 g/L $NH_4Cl$, 5 g/L $KH_2PO_4$, and 0.8 g/L $MgSO_4{\cdot}7H_2O$ was used in this study. Glucose (40 g/L) was added to M medium to produce MD medium. Xylose (15 g/L) was added to M medium to produce MX medium. The addition of glucose (40 g/L) and xylose (25 g/L) to M medium produced MDX medium. No other nutrients, including sources of nitrogen, vitamins, and trace elements, were added to these media (MD, MX, and MDX). The pHs of these media were adjusted to 5.0 with 1 M $CaCO_3$.

### Lignocellulosic hydrolysate

Japanese cedar wood chips (a gift of Dr. Tomoaki Minowa (AIST)) were mechanochemically treated and then enzymatically hydrolyzed, as reported previously [21]. In the enzymatic hydrolysis assay (72 h, 50 °C), 200 mL of the cellulose solution [50 FPU/g-dry substrate Acremozyme (Meiji Co., Ltd, Tokyo, Japan) and 20 μl/g dry substrate Optimash BG (Genencor International, Rochester, NY, USA)] in 50 mM citrate buffer (pH 5.0) was added to 40 g of pretreated materials. The resulting hydrolysate was used as lignocellulosic hydrolysate supplemented for aerobic

cultivation performance analysis. The wood chip hydrolysate contained 70.3 g/L glucose, 7.4 g/L xylose, 10.5 g/L mannose, and 1.2 g/L galactose; these values were determined as described below.

### Aerobic batch cultivation

*hap4* deletion strain B42-DHAP4 and the control strain MA-B42 were first cultivated aerobically in 6 mL YPD medium for 36 h at 30 °C. Then, a 2-mL aliquot of pre-culture was transferred to 100 mL YPD medium in a 300 mL Erlenmeyer flask (with a silicone sponge closure) and cultivated aerobically with a rotation speed of 116 rpm for 36 h at 30 °C. The culture was centrifuged at 6000×*g* for 5 min at 4 °C, and the pelleted cells were washed and resuspended in distilled water. The washed cells were inoculated into cultivation medium. For all cultivation media, the initial cell density was adjusted to approximately 3.16 g [dry cell weight (DCW)]/L. For aerobic batch cultivation, 50 mL cultures in cultivation medium (MD, MX, and MDX) and 10 mL lignocellulosic hydrolysates were grown in 300 and 100 mL Erlenmeyer flasks (with silicone sponge closures), respectively. Aerobic batch cultivations were performed at 30 °C with 116 rpm agitation in a rotary shaker (SCS-40N; Sankiseiki, Osaka, Japan). Samples (0.3 mL) of the cultivation broth (MD, MX, and MDX media) were removed at specified intervals and diluted fourfold with 8 mM $H_2SO_4$. Samples (0.1 mL) of the lignocellulosic hydrolysate were removed at specified intervals and diluted tenfold with distilled water. These diluted samples were stored at −30 °C for high-performance liquid chromatography (HPLC) analysis of substrates and cultivation products.

### Analytical methods

Dry cell weight was determined by measuring the absorbance of diluted culture samples at 600 nm using a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan), as described previously [20]. Substrates consumed and products formed were analyzed with an HPLC system equipped with a refractive index detector (RI-2031Plus; JASCO, Tokyo, Japan). Ethanol, glucose, xylose, acetate, pyruvate, glycerol, and xylitol in the cultivation media (MD, MX, and MDX) were separated using an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA). The HPLC apparatus was operated at 65 °C with 5 mM $H_2SO_4$ at a flow rate of 0.6 mL/min as the mobile phase. Cultivation metabolites in lignocellulosic hydrolysates were separated by an Aminex HPX-87P column (Bio-Rad), as reported previously [22]. The HPLC system was operated at 70 °C with sterile water (flow rate, 0.6 mL/min) as the mobile phase.

## Results and discussion

### Effect of *hap4* deletion on ethanol production from glucose

To assess the effect of complete respiratory deficiency on ethanol production and growth characteristics of a xylose-utilizing *S. cerevisiae* strain, the completely respiration-deficient *hap4*Δ strain (B42-DHAP4) and a control strain (MA-B42) were constructed. These recombinant strains had similar (high) activities of XR, XDH, and XK (data not shown). In addition, we noted that strain B42-DHAP4 could not grow on glycerol, a non-fermentable carbon source (data not shown), suggesting that the electron transfer chain in the mitochondria was disrupted in the *hap4* mutant. Ethanol production from glucose by MA-B42 and B42-DHAP4 was first compared using 40 g/L glucose as the sole carbon source (MD medium) (Fig. 1a, b). After 48 h of cultivation, the cultures of these two strains yielded approximately 4.47 g/L DCW for MA-B42 and 3.93 g/L DCW for B42-DHAP4 (Fig. 1a, b). Biomass yields calculated from the cell densities of B42-DHAP4 were 11 % lower than those of MA-B42 in cultivation using MD medium (Table 1). Thus, lower biomass concentrations and biomass yields from glucose were observed with B42-DHAP4 compared to MA-B42, which is in good agreement with previous studies using *hap4*Δ strains [16, 23]. MA-B42 depleted the available glucose within 48 h (Fig. 1a), whereas B42-DHAP4 consumed glucose more slowly; glucose was almost completely depleted by B42-DHAP4 within 72 h (Fig. 1b); B42-DHAP4 produced a maximum of 15.7 g/L ethanol at 55 h (Fig. 1b), while peak ethanol production by MA-B42 remained less than 14.6 g/L (Fig. 1a). MA-B42 consumed more ethanol than B42-DHAP4 upon depletion of glucose (after 48 h of cultivation) (Fig. 1a, b), which may partially explain better ethanol production performance in B42-DHAP4 than in MA-B42. As shown in Table 1, the maximum specific glucose consumption rate was not statistically distinguishable between the two strains, while the ethanol production rate was 14 % higher in B42-DHAP4 than in MA-B42. The ethanol yield of B42-DHAP4 on glucose was 10 % higher than that of MA-B42 (Table 1), consistent with a previous finding that *hap4*Δ mutants displayed increased ethanol yields on glucose [16, 23]. The pyruvate and glycerol yields of B42-DHAP4 on glucose were higher than those of MA-B42 (Table 1). Meanwhile, B42-DHAP4 had a 63 % lower acetate yield than that of MA-B42 (Table 1). A similar trend for by-product yield also was observed in a previous report [23]. Thus, the biomass, ethanol, and by-product production performance on glucose as

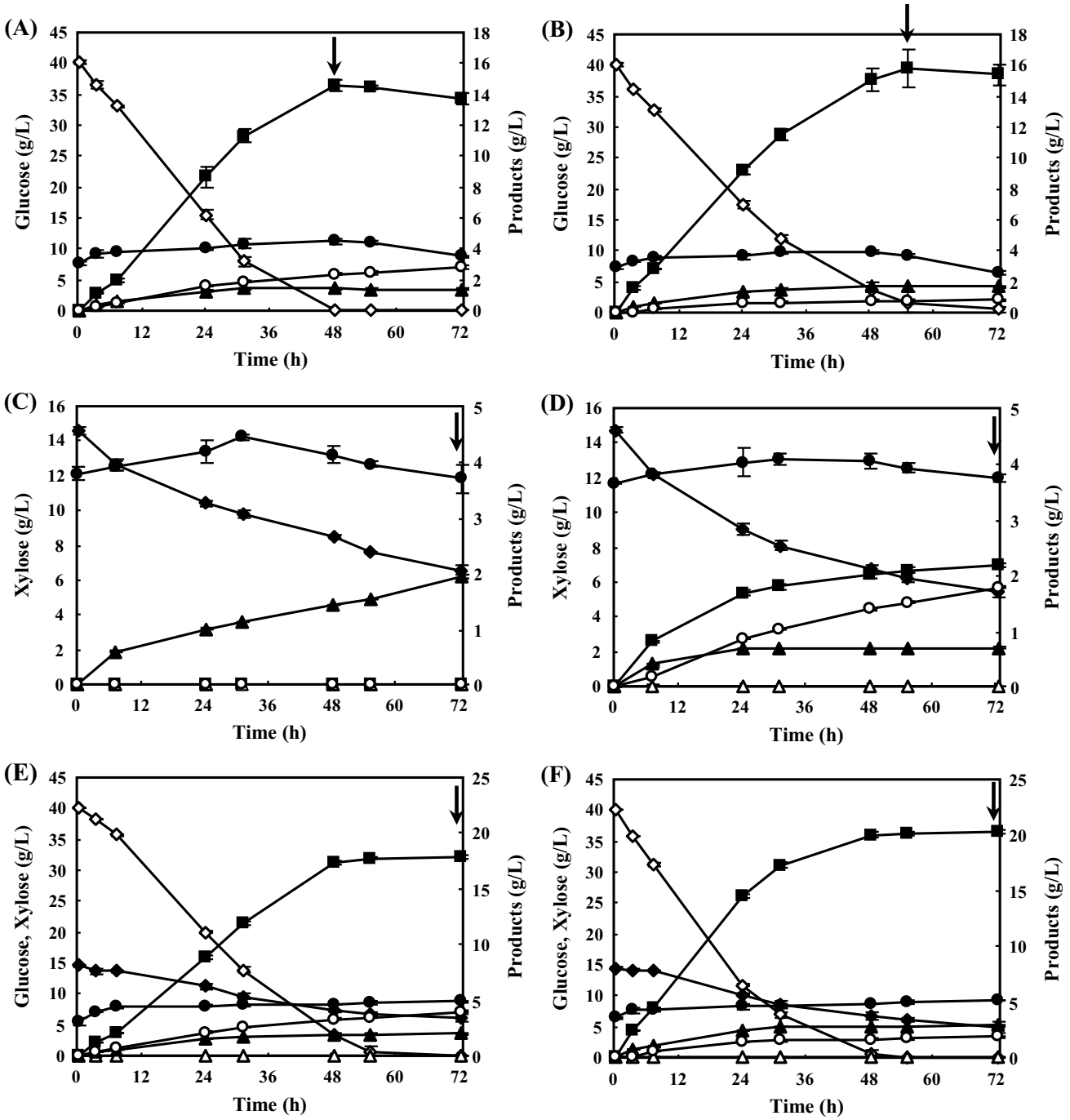


**Fig. 1** Time-dependent batch cultivation profiles of MA-B42 (**a**, **c**, **e**) and B42-DHAP4 (**b**, **d**, **f**) grown under aerobic conditions in MD medium (containing 40 g/L glucose) (**a**, **b**), MX medium (containing 15 g/L xylose) (**c**, **d**), and MDX medium (containing 40 g/L glucose and 25 g/L xylose) (**e**, **f**). Glucose, *open diamonds*; xylose, *closed diamonds*; ethanol, *closed squares*; xylitol, *open triangles*; glycerol, *closed triangles*; acetate, *open circles*; dry cell weight, *closed circles*. The mean values and standard deviations (*error bars*) from three independent experiments are presented. The *arrows* indicate time points at which the yields of products were calculated and described in Table 1

the substrate (e.g., reduced biomass and increased ethanol yields) is similar among other respiration-deficient *hap4*Δ strains and the xylose-utilizing B42-DHAP4 strain used in the present study.

**Table 1** Comparison of fermentation performance of *S. cerevisiae* strains MA-B42 and B42-DHAP4 in aerobic batch cultivations with MD, MDX, and MX media

| Strain | Medium | Max. specific glucose consumption rate (g-glucose/g-DCW/h) | Max. specific xylose consumption rate (g-xylose/g-DCW/h) | Max. specific ethanol production rate (g-ethanol/g-DCW/h) | Biomass yield (g-DCW/g-consumed total sugar) | Ethanol yield (g-ethanol/g-consumed total sugar) | Acetate yield (g-acetate/g-consumed total sugar) | Pyruvate yield (g-pyruvate/g-consumed total sugar) | Glycerol yield (g-glycerol/g-consumed total sugar) |
|---|---|---|---|---|---|---|---|---|---|
| MA-B42 | MD | 0.241 ± 0.012 | ND | 0.084 ± 0.005 | 0.112 ± 0.005 | 0.366 ± 0.004 | 0.059 ± 0.003 | 0.008 ± 0.001 | 0.036 ± 0.000 |
| | MX | ND | 0.070 ± 0.005 | ND | 0.554 ± 0.008 | ND | ND | 0.099 ± 0.004 | 0.241 ± 0.009 |
| | MDX | 0.186 ± 0.002 | 0.036 ± 0.002 | 0.085 ± 0.001 | 0.122 ± 0.003 | 0.368 ± 0.000 | 0.081 ± 0.001 | ND | 0.040 ± 0.001 |
| B42-DHAP4 | MD | 0.233 ± 0.004 | ND | 0.096 ± 0.003 | 0.100 ± 0.002 | 0.403 ± 0.020 | 0.022 ± 0.001 | 0.017 ± 0.001 | 0.043 ± 0.001 |
| | MX | ND | 0.095 ± 0.003 | 0.030 ± 0.001 | 0.447 ± 0.012 | 0.233 ± 0.005 | 0.191 ± 0.003 | 0.045 ± 0.001 | 0.075 ± 0.001 |
| | MDX | 0.261 ± 0.019 | 0.041 ± 0.006 | 0.133 ± 0.012 | 0.127 ± 0.001 | 0.408 ± 0.004 | 0.038 ± 0.000 | ND | 0.057 ± 0.001 |

Values are the means ± standard deviations from three independent experiments. Time points used for product yield calculations are indicated in Fig. 1

*ND* not detectable

### Effect of *hap4* deletion on ethanol production from xylose

We next examined possible effects of *hap4*Δ cells on ethanol production from xylose. MA-B42 and B42-DHAP4 were cultivated in MX medium containing xylose (15 g/L) (Fig. 1c, d). During these experiments, both of the recombinant strains grew weakly on xylose as the sole carbon source (Fig. 1c, d). This result is similar to previous observations in which the XR/XDH/XK-expressing strains and petite (respiration-deficient) mutants did not grow on xylose plates [5, 15]. After a 31-h cultivation in MX medium, the maximum cell densities of MA-B42 and B42-DHAP4 yielded 4.44 g/L DCW and 4.08 g/L DCW, respectively (Fig. 1c, d). At 72 h, MA-B42 consumed 8.01 g/L xylose and produced glycerol as a major product and no ethanol was formed (Fig. 1c), whereas B42-DHAP4 consumed 9.30 g/L xylose and produced 2.17 g/L ethanol (Fig. 1d). The reason for the higher glycerol production of MA-B42 is unclear at present. However, it is likely to be highly correlated with the activity of the glycerol 3-phosphate (G3P) shuttle systems, a redox adjustment mechanism under aerobic conditions [24]. The specific xylose consumption rate was 36 % higher in B42-DHAP4 than in MA-B42 (Table 1). It was surprising that B42-DHAP4 had the ability to produce ethanol from xylose even under aerobic conditions, although the production rate and yield of ethanol obtained with B42-DHAP4 were markedly lower (by 3.2-fold and 42 %, respectively) during cultivation in MX medium compared to those obtained during cultivation in MD medium (Table 1). In contrast to the results seen in MD medium, the pyruvate and glycerol yields of B42-DHAP4 were lower than those obtained for MA-B42 grown in MX medium (Table 1). Notably, the glycerol yield for B42-DHAP4 was 69 % lower than that for MA-B42, an observation that may be directly related to the enhanced aerobic ethanol production from xylose by B42-DHAP4. Meanwhile, the acetate yield for B42-DHAP4 increased compared to that obtained for MA-B42 (Table 1). Interestingly, no xylitol was detected in the cultures of MA-B42 and B42-DHAP4 (Fig. 1c, d). Since xylose metabolism in recombinant *S. cerevisiae* is fully oxidative [5, 6], metabolic flux at the pyruvate branch point in MA-B42 favored respiration over ethanol production. Conversely, inhibition of respiration by deleting *HAP4* increases the metabolic flux into ethanol production, probably due to the blocking of terminal NADPH oxidation by respiration. Thereby, the respiration-deficient B42-DHAP4 strain exhibited efficient aerobic production of ethanol from xylose.

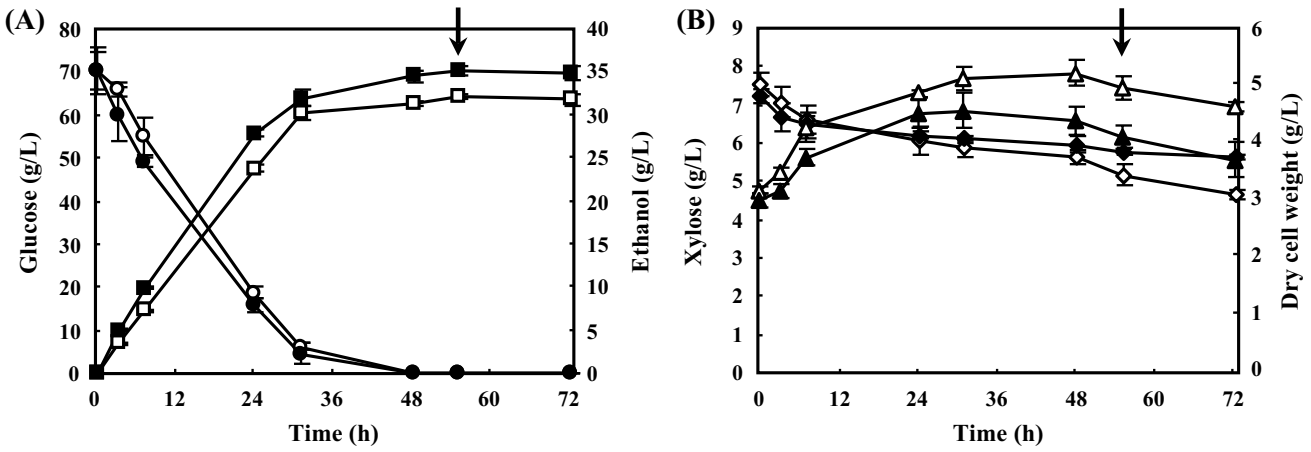


**Fig. 2** Time-dependent batch cultivation profiles of glucose consumption and ethanol production (**a**) or xylose consumption and dry cell weight (**b**) by MA-B42 (*open symbol*) and B42-DHAP4 (*closed symbol*) grown in lignocellulosic hydrolysate under aerobic conditions. Glucose, *circles*; ethanol, *squares*; xylose, *diamonds*; dry cell weight, *triangles*. The mean values and standard deviations (*error bars*) from four independent experiments are presented. The *arrows* indicate time points at which the yields of products were calculated and described in Table 2

### Effect of *hap4* deletion on ethanol production from sugar mixture

Ethanol production by MA-B42 and B42-DHAP4 from a sugar mixture was assayed using 40 g/L glucose and 25 g/L xylose as carbon sources (MDX medium) (Fig. 1e, f). There was no apparent difference in the cell growth (Fig. 1e, f) and biomass yield (Table 1) between these recombinant yeast strains, and the cultures yielded approximately 4.97 g/L DCW after 72 h of cultivation (Fig. 1e, f). Although both strains depleted the glucose supply within 72 h (Fig. 1e, f), the maximum specific glucose consumption rate was approximately 40 % higher in B42-DHAP4 compared to MA-B42 (Table 1). Compared to the cultivation results obtained using MD medium, B42-DHAP4 consumed glucose more rapidly in the presence of xylose (see Fig. 1b, f; Table 1). In contrast to B42-DHAP4, the specific glucose consumption rates of MA-B42 were 23 % lower for the mixture of glucose and xylose than for glucose alone (see Fig. 1a, e; Table 1), although the amount of cells capable of consuming glucose in the mixture of glucose and xylose was greater than in glucose alone. It should be noted that the repression of glucose utilization in the presence of xylose was not observed in other recombinant industrial *S. cerevisiae* strains carrying the same genes on their chromosomes [25], suggesting a strain-dependent effect. Another possible reason for the slower consumption of glucose in the presence of xylose is that there is competition for the unspecific hexose transporters mediating uptake of xylose in MA-B42. On the other hand, at the end of cultivation, approximately 54 and 48 % of the initial xylose remained unconsumed with MA-B42 and B42-DHAP4, respectively (Fig. 1e, f). B42-DHAP4 demonstrated a 14 % higher rate of xylose consumption than MA-B42 (Table 1). As a result, the rate of maximum specific ethanol production achieved by B42-DHAP4 was approximately 56 % higher than that achieved by MA-B42 (Table 1). After 72 h of cultivation, the peak ethanol levels produced by MA-B42 and B42-DHAP4 were 17.9 and 20.2 g/L, respectively (Fig. 1e, f). Moreover, B42-DHAP4 had 11 % higher ethanol yield than MA-B42 (Table 1), and the ethanol yields of MA-B42 and B42-DHAP4 corresponded to 72.0 and 79.8 % of the theoretical yield, respectively. Compared to MA-B42, acetate yield was decreased and glycerol yield was slightly increased in the B42-DHAP4 strain (Table 1). Thus, the cultivation performance of B42-DHAP4 was significantly improved, with increases in the production rate and yield of ethanol compared with those of MA-B42 during cultivation in the mixed glucose and xylose (MDX medium). The higher ethanol yield of B42-DHAP4 apparently correlated directly with low acetate yield. As seen during the cultivation in MX medium, xylitol was not produced in either strain during cultivation in MDX (Fig. 1e, f). Based on the result from MX medium, the enhanced ethanol production by deletion of *HAP4* during cultivation in the glucose/xylose mixture may be mainly ascribed to the increased production of ethanol from xylose under aerobic conditions.

### Effect of *hap4* deletion on ethanol production from lignocellulosic hydrolysate

Finally, on the basis of the above results, we investigated whether B42-DHAP4 indeed efficiently produced ethanol

from the mixture of sugars (including glucose and xylose) present in lignocellulosic hydrolysate. B42-DHAP4 and control strain MA-B42 were cultivated in a lignocellulosic Japanese cedar-based hydrolysate that contained 70.3 g/L glucose and 7.4 g/L xylose. The cell densities of MA-B42 and B42-DHAP4 increased gradually during cultivation, with peak yields of 5.20 g/L DCW after 48 h and 4.55 g/L DCW after 31 h, respectively (Fig. 2b). Biomass yields calculated from the cell concentration of B42-DHAP4 were 15 % lower than that of MA-B42 (Table 2). Thus, as with the cultivation in glucose-containing (MD) medium, B42-DHAP4 formed significantly less biomass than MA-B42 during cultivation in lignocellulosic hydrolysate. Both MA-B42 and B42-DHAP4 depleted the glucose supply within 48 h (Fig. 2a), but B42-DHAP4 exhibited a 24 % higher maximum specific glucose consumption rate compared to that of the control strain MA-B42, consistent with the results from cultivation with MDX medium (Table 2). On the other hand, MA-B42 and B42-DHAP4 converted xylose very slowly (Fig. 2b), consuming only 38 and 22 % of the xylose after 72 h, respectively. The rate of maximum specific xylose consumption achieved during cultivation in lignocellulosic hydrolysate was approximately 75–85 % lower than that observed in MDX (compare Tables 1, 2). Moreover, neither mannose nor galactose was fully depleted by either of the two strains within 72 h (data not shown); only 53 % of the mannose and 26 % of the galactose were consumed by either strain after 72 h. These results may be partially due to a growth-inhibitory effect of the components of lignocellulosic hydrolysate. In fact, the lignocellulosic hydrolysate used in this study contained 1.7 g/L acetate, a weakly acidic growth inhibitor known to be generated by the deacetylation of hemicellulose during the pretreatment of lignocellulosic biomass [26]. It also should be noted that the xylose consumption rate is more strongly affected by the presence of acetate and low pH than the glucose consumption rate [27]. In addition, we used a laboratory strain (BY4742) as a host for genetic manipulation in this study; laboratory strains typically exhibit a less rapid fermentation rate and lower tolerance to inhibitory substances than do industrial strains. This difference may contribute to the limited capacity for sugar consumption in MA-B42 and B42-DHAP4.

The peak ethanol concentrations produced by B42-DHAP4 and MA-B42 were 35.0 and 32.1 g/L, respectively, and were achieved after 55 h of cultivation (Fig. 2a). B42-DHAP4 had a 27 % higher rate of maximum specific ethanol production than MA-B42 (Table 2). Furthermore, the ethanol yield of B42-DHAP4 (86.6 % of the theoretical yield) was higher than that of MA-B42 (78.8 %) (Table 2). Thus, superior maximum concentration, production rate, and yield of ethanol were achieved by B42-DHAP4 compared to MA-B42 when grown on

**Table 2** Comparison of fermentation performance of *S. cerevisiae* strains MA-B42 and B42-DHAP4 in aerobic batch cultivations with lignocellulosic hydrolysate

| Strain | Max. specific glucose consumption rate (g-glucose/g-DCW/h) | Max. specific xylose consumption rate (g-xylose/g-DCW/h) | Max. specific ethanol production rate (g-ethanol/g-DCW/h) | Biomass yield (g-DCW/g-consumed total sugar) | Ethanol yield (g-ethanol/g-consumed total sugar) | Acetate yield (g-acetate/g-consumed total sugar) | Glycerol yield (g-glycerol/g-consumed total sugar) |
|---|---|---|---|---|---|---|---|
| MA-B42 | 0.445 ± 0.040 | 0.009 ± 0.001 | 0.204 ± 0.004 | 0.067 ± 0.004 | 0.403 ± 0.028 | 0.018 ± 0.003 | 0.011 ± 0.001 |
| B42-DHAP4 | 0.551 ± 0.021 | 0.006 ± 0.001 | 0.259 ± 0.018 | 0.057 ± 0.004 | 0.443 ± 0.020 | 0.008 ± 0.001 | 0.015 ± 0.001 |

Values are the means ± standard deviations from four independent experiments. Time points used for product yield calculations are indicated in Fig. 2

*ND* not detectable

lignocellulose hydrolysate, as also seen for growth on MDX medium. Acetate and glycerol accumulation in the lignocellulose hydrolysate medium was observed for both recombinant strains (no more than 1.5 and 1.2 g/L, respectively), whereas xylitol and pyruvate accumulation was not observed with either strain. Compared with MA-B42, B42-DHAP4 showed a greater than twofold lower acetate yield (Table 2). In contrast, the glycerol yield was approximately 36 % higher for B42-DHAP4 than for MA-B42 (Table 2). The lower acetate yield of B42-DHAP4 may be directly related to the high ethanol yield. These results indicated that mixed sugars present in the lignocellulosic hydrolysate can be efficiently converted to ethanol by the B42-DHAP4 strain, in which *hap4* was disrupted. Thus, this study successfully demonstrates the utility of using a respiratory-deficient *hap4*Δ strain for the bioconversion of lignocellulosic hydrolysate to ethanol.

## Conclusion

The *S. cerevisiae HAP4* gene encodes a transcription factor involved in the control of respiration. We investigated the effects of *hap4* deletion on aerobic ethanol and biomass production by a xylose-utilizing *S. cerevisiae* strain growing on medium containing glucose and/or xylose. For each of the tested cultivation conditions, ethanol production was enhanced in the *hap4* deletion strain (B42-DHAP4). We also demonstrated that B42-DHAP4 had the ability to produce ethanol from xylose as a single carbon source even under aerobic conditions. Furthermore, B42-DHAP4 showed a higher ethanol yield (0.443 g/g of total sugars) and a higher maximum specific ethanol production rate (0.259 g-ethanol/g-DCW/h) than the control strain (MA-B42) when cultured on detoxified hydrolysate of wood chips. To our knowledge, this is the first report of effective ethanol production not only from xylose but also from the mixed sugars present in lignocellulosic hydrolysates using a *hap4* deletion strain with xylose-utilizing ability. The results of this study suggest that *hap4* deletion in engineered yeast strains will facilitate aerobic ethanol production from hydrolysates of lignocellulosic biomass.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no competing interests.

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