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Effect of Initial Cell Concentration on Ethanol Production by Flocculent *Saccharomyces cerevisiae* with Xylose-Fermenting Ability

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Abstract Different initial cell concentrations of a recombinant flocculent *Saccharomyces cerevisiae* MA-R4 were evaluated for their effects on xylose fermentation and glucose–xylose cofermentation. A high initial cell concentration greatly increased both the substrate utilization and ethanol production rates. During xylose fermentation, the highest rates of xylose consumption (2.58 g/L h) and ethanol production (0.83 g/L h) were obtained at an initial cell concentration of 13.1 g/L. During cofermentation, the highest rates of glucose consumption (14.4 g/L h), xylose consumption (2.79 g/L h), and ethanol production (6.68 g/L h) were obtained at an initial cell concentration of 12.7 g/L. However, a high initial cell density had no positive effect on the maximum ethanol concentration and ethanol yield mainly due to the increased amount of by-products including xylitol. The ethanol yield remained almost constant (0.34 g/g) throughout xylose fermentation (initial cell concentration range, 1.81–13.1 g/L), while it was slightly lower at high initial cell concentrations (9.87 and 12.7 g/L) during cofermentation. The determination of the appropriate initial cell concentration is necessary for the improvement of substrate utilization and ethanol yield.

Keywords Recombinant *Saccharomyces cerevisiae* · Xylose · Ethanol · Cofermentation · Cell concentration

Introduction

Lignocellulosic biomass from agricultural and forest residues represents an attractive renewable feedstock for bioethanol production. It contains large amounts of potentially fermentable sugars in the form of cellulose and hemicellulose. To convert lignocellulosic biomass into useable energy that is economical and environmentally friendly, both cellulose and hemicellulose must be used. Cellulose is a highly crystalline polymer of glucose, while hemicellulose is a branched polymer that can be hydrolyzed into hexose sugars (i.e.,

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glucose, galactose, and mannose) and pentose sugars (i.e., xylose and arabinose) [1]. Xylose is the dominant pentose sugar in hemicellulose hydrolysates and, as such, is the second most abundant carbohydrate in nature following glucose [1]. Consequently, xylose utilization is important for successful biomass-ethanol fermentation. On the other hand, xylulose, an intermediate product of xylose, is a natural substrate of *Saccharomyces cerevisiae* and enters the pentose phosphate pathway as xylulose 5-phosphate.

The yeast *S. cerevisiae* is traditionally used for industrial ethanol production owing to its high ethanol productivity and high tolerance to ethanol. Furthermore, *S. cerevisiae* is relatively resistant to low pH and lignocellulose-derived inhibitors [2]. However, *S. cerevisiae* cannot ferment xylose due to its lack of an active catabolic pathway for this sugar. Although many attempts have been made to develop recombinant *S. cerevisiae* strains that can ferment xylose [2–7], there are still some fundamental problems linked to its industrial use. Recently, we [8] have reported the development of a recombinant industrial *S. cerevisiae* strain MA-R4 that can efficiently coferment glucose and xylose to ethanol and has high ethanol productivity. The MA-R4 strain was engineered by chromosomal integration to express genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Pichia stipitis* along with *S. cerevisiae* xylulokinase (XK) gene using the alcohol-fermenting flocculent yeast strain IR-2. IR-2 has the highest xylulose-fermenting ability among many different industrial diploid strains, suggesting that with its industrial background IR-2 is a useful host strain for genetically engineered xylose-utilizing *S. cerevisiae* [9].

Optimizing fermentation conditions such as substrate and initial cell concentrations is important for obtaining the maximum rate of ethanol production and maximum yield of ethanol from xylose. Previous studies have shown that the initial cell concentration not only influences cellular and molecular behaviors [10, 11] but also changes the lag phase of cell growth, growth and fermentation rates, final product concentration, and yield [12–15]. This study examined the effects of the initial cell concentration of MA-R4 on fermentation parameters using complex medium containing xylose or mixtures of glucose and xylose.

Materials and Methods

Microorganism and Media

The xylose-utilizing recombinant *S. cerevisiae* strain MA-R4, derived from a diploid and flocculent yeast strain IR-2 [16], was used in this study. MA-R4 was genetically engineered with chromosome-integrated XR, XDH, and XK genes under the control of the *PGK* promoter [8, 9]. Briefly, plasmid pAUR-XKXDHXR [17] digested with the restriction enzyme *Bsi*WI was chromosomally integrated into the *aur1* locus of IR-2 to construct the recombinant strain MA-R4. This strain was maintained by selective growth on yeast peptone (YP) medium (10 g/L yeast extract and 20 g/L peptone) supplemented with 20 g/L glucose (YPD medium) in the presence of 0.5 mg/L aureobasidin A (Takara Bio, Kyoto, Japan). Xylose (45 g/L) was added to YP medium to produce YPX medium. The addition of glucose (45 g/L) and xylose (45 g/L) to YP medium produced YPDX medium. The YPX and YPDX media were used as the fermentation media in this study.

Inoculum Preparation and Fermentation

For inoculum preparation, MA-R4 was aerobically cultivated in 5 mL YPD medium for 16 h at 30 °C. Then, a 2-mL aliquot of pre-culture was transferred to 100 mL YPD medium

in 300-mL Erlenmeyer flasks with silicone sponge closures and cultivated aerobically with a rotation speed of 116 rpm for 36 h at 30 °C. The culture was centrifuged at 5,000 rpm for 5 min at 4 °C, and the supernatant was discarded. The precipitated cells were washed and resuspended in distilled water to give a cell concentration of approximately 135.4 g (dry cell weight, DCW)/L. These cells were diluted to designated concentrations and then inoculated into 20 mL fermentation medium (YPX or YPDX). The initial cell concentrations employed to start fermentation in YPX medium were 1.81, 3.36, 6.78, 9.81, and 13.1 g/L, while those employed to start fermentation in YPDX medium were 1.60, 3.27, 6.76, 9.87, and 12.7 g/L. Anaerobic batch fermentations were performed at 30 °C in sterile closed bottles (50 mL) with magnetic stirring as described previously [8, 9, 17]. Samples (0.3 mL) of fermentation broth (YPX and YPDX) were removed at specified intervals and diluted four times with 8 mM H_2SO_4. These diluted samples were stored at -30 °C for high-performance liquid chromatography (HPLC) analysis of substrates and fermentation products. All experiments were performed in triplicate.

Analytical Methods

DCW (namely, cell concentration) was determined using a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan) which measured the absorbance of the samples at 600 nm, as described previously [17]; an optical density of 1 is approximately equal to 0.13 g of DCW/L. Concentrations of glucose, xylose, ethanol, xylitol, glycerol, and acetic acid were determined by HPLC (Jasco, Tokyo, Japan) equipped with a refractive index detector (RI-2031Plus; JASCO) using an Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA, USA) and Cation H refill guard (Bio-Rad) column. The HPLC apparatus was operated at 65 °C, with 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 Initial Cell Concentration on Xylose Fermentation

First, the effect of initial cell concentration on xylose-to-ethanol bioconversion was investigated using recombinant *S. cerevisiae* strain MA-R4. Figure 1 shows the cell, xylose, and ethanol concentration profiles during xylose batch fermentation for various initial cell concentrations ranging from 1.81 to 13.1 g/L MA-R4. The concentration of MA-R4 at different initial cell concentrations (1.81, 3.36, 6.78, 9.81, and 13.1 g/L) increased gradually during fermentation, reaching 6.78, 6.12, 9.27, 12.2, and 15.4 g/L after 72 h fermentation, respectively (Fig. 1a). The amount of xylose consumed after 24 h of fermentation was 12.5, 18.4, 31.9, 39.3, and 41.6 g/L for initial cell concentrations of 1.81, 3.36, 6.78, 9.81, and 13.1 g/L, respectively (Fig. 1b), resulting in increases in the amount of consumed xylose as the initial cell concentration was increased. For all initial cell concentrations, xylose was almost completely consumed after 72 h of fermentation (Fig. 1b). The concentration of ethanol produced after 24 h of fermentation was 3.25, 5.77, 10.6, 13.1, and 14.3 g/L for the initial cell concentrations of 1.81, 3.36, 6.78, 9.81, and 13.1 g/L, respectively (Fig. 1c), showing a trend similar to xylose consumption (increased ethanol concentration at higher initial cell concentrations), most likely due to the increased number of cells available to convert xylose to ethanol. Table 1 shows these improved rates of xylose consumption and ethanol production with increasing initial cell concentrations. These results are consistent

with a previous finding that a high initial cell concentration of *P. stipitis* can increase the rate of xylose utilization and ethanol formation [14]. The highest rates for xylose consumption (2.58 g/L h) and ethanol production (0.83 g/L h) were achieved at an initial cell concentration of 13.1 g/L MA-R4, although the maximum ethanol concentration was almost the same in all cases (Table 1). Thus, the rate of xylose consumption and ethanol production during fermentation may depend on the amount of cells present in the initial inoculum.

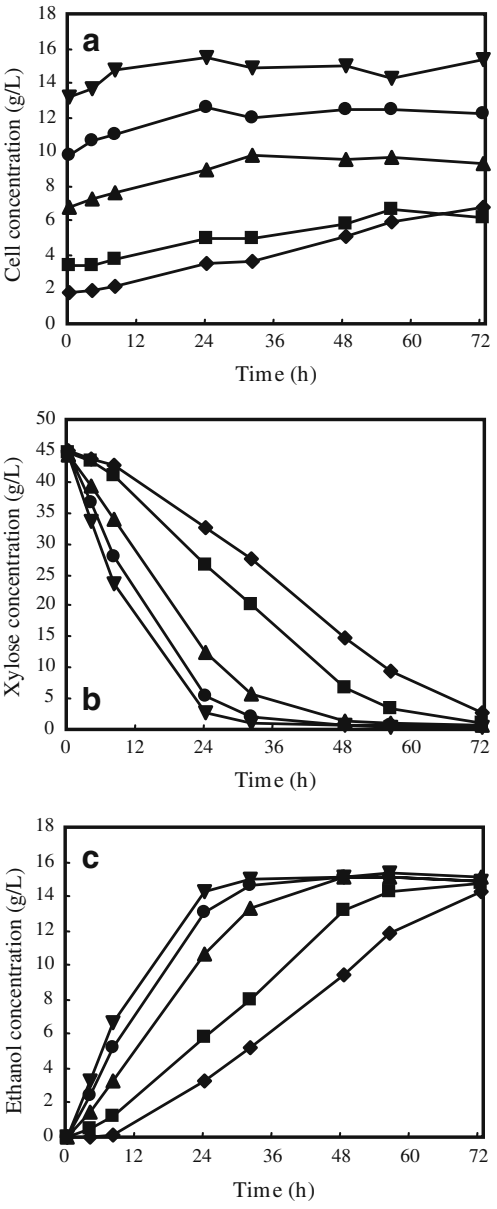

Fig. 1 Time-dependent ethanol fermentation profiles of **a** cell, **b** xylose, and **c** ethanol concentrations at different initial cell concentrations of recombinant *S. cerevisiae* strain MA-R4 in YPX medium containing xylose (45 g/L). ♦, 1.81 g/L; ■, 3.36 g/L; ▲, 6.78 g/L; ●, 9.81 g/L; ▼, 13.1 g/L initial cells. Values are averages from three independent experiments

Table 1 Summary of the 72-h fermentation of xylose by *S. cerevisiae* strain MA-R4.

Parameter	Initial cell concentration (g/L)				
	1.81±0.08	3.36±0.30	6.78±0.53	9.81±0.86	13.1±1.16
Initial xylose concentration (g/L)	44.6±0.1	44.8±0.8	44.4±0.3	44.6±1.2	44.2±0.8
Maximum ethanol concentration (g/L)	14.2±0.52	14.8±0.09	15.4±0.13	15.2±0.27	15.1±0.10
Maximum xylose consumption rate (g/L h)	0.633±0.027	0.791±0.044	1.328±0.073	1.637±0.040	2.579±0.191
Maximum ethanol production rate (g/L h)	0.212±0.018	0.274±0.028	0.441±0.020	0.546±0.031	0.827±0.076
Ethanol yield (g/g)	0.340±0.006	0.335±0.012	0.337±0.013	0.333±0.011	0.334±0.015
Xylitol yield (g/g)	0.049±0.015	0.053±0.009	0.057±0.006	0.070±0.005	0.077±0.007
Glycerol yield (g/g)	0.111±0.001	0.109±0.008	0.122±0.007	0.114±0.018	0.110±0.016
Acetic acid yield (g/g)	0.018±0.002	0.019±0.001	0.025±0.001	0.024±0.002	0.023±0.002

Values are averages of three independent experiments ± standard deviation

Figure 2 shows the maximum production of by-products (xylitol, glycerol, and acetic acid) at different initial cell concentrations. At initial cell concentrations of 1.81, 3.36, 6.78, 9.81, and 13.1 g/L, the amount of xylitol produced was 2.13, 2.27, 2.60, 3.03, and 3.24 g/L, respectively; that of glycerol was 4.14, 4.23, 4.77, 4.71, and 4.60 g/L, respectively; and that of acetic acid was 0.65, 0.74, 1.02, 1.07, and 1.11 g/L, respectively. While the concentration of xylitol linearly increased with increasing initial cell concentrations, those of glycerol and acetic acid increased only at initial cell concentrations up to 6.78 g/L and did not markedly increase at concentrations higher than 6.78 g/L. As a result, the xylitol yield increased with increasing initial cell concentrations, the glycerol yield was relatively constant except for the initial cell concentration of 6.78 g/L, and the acetic acid yield increased with increasing initial cell concentrations from 3.36 to 6.78 g/L and was relatively constant from 6.78 to 13.1 g/L (Table 1). However, in the case of ethanol, no significant difference in yield was observed over the initial cell concentration range (0.34, 0.34, 0.34, 0.33, and 0.33 g/L for

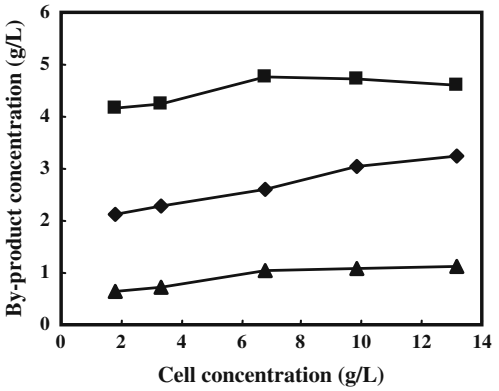

Fig. 2 Effect of initial cell concentration on the maximum production of xylitol (♦), glycerol (■), and acetic acid (▲) from xylose fermentation by recombinant *S. cerevisiae* strain MA-R4. Values are averages from three independent experiments

initial cell concentrations of 1.81, 3.36, 6.78, 9.81, and 13.1 g/L, respectively). The reason for this constant ethanol yield may be attributed to the neutralizing effect of two influential factors: an increased yield owing to less cell biomass production and a decreased yield owing to by-product formation.

Effect of Initial Cell Concentration on Glucose–Xylose Cofermentation

As both glucose and xylose are present in lignocellulosic hydrolysates, the batch fermentation behavior of recombinant *S. cerevisiae* MA-R4 at different initial cell concentrations (1.60 to 12.7 g/L) was also examined using a 1:1 glucose–xylose mixture. The profiles for cell, glucose, and xylose concentration, as well as ethanol concentration, are shown in Fig. 3. Although MA-R4 utilized glucose more rapidly than xylose, this strain could simultaneously coferment glucose and xylose at any initial cell concentration (0 to 9 h; Fig. 3b), which is consistent with our previous findings [8]. MA-R4 grew quickly during glucose and xylose cofermentation (0 to 9 h; Fig. 3a) but hardly grew during the xylose-only consumption phase (9 to 48 h; Fig. 3a). After 48 h of cofermentation, the initial cell concentrations (1.60, 3.27, 6.76, 9.87, and 12.7 g/L) increased to 13.8, 12.2, 15.8, 17.4, and 17.0 g/L, respectively (Fig. 3a). At these initial cell concentrations, the amount of glucose consumed after 3 h of fermentation was 10.9, 19.0, 31.9, 39.7, and 43.3 g/L, respectively (Fig. 3b), while the amount of xylose consumed after 24 h of fermentation was 37.0, 37.1, 40.4, 41.8, and 42.6 g/L, respectively. Thus, the amount of consumed sugars (glucose and xylose) increased with increasing initial cell concentrations. The consumption rate of glucose and xylose was also enhanced by increasing the initial cell concentration (Table 2). The highest rates of glucose (14.4 g/L h) and xylose (2.79 g/L h) consumption were achieved with the initial cell concentration of 12.7 g/L MA-R4. However, a comparison of Tables 1 and 2 shows that the xylose consumption rate during glucose–xylose cofermentation was not markedly different from that during xylose fermentation. At all initial cell concentrations, glucose was completely consumed within 9 h, and almost all xylose was metabolized after 48 h fermentation (Fig. 3b). At the end of glucose fermentation, 24.9–36.4% of xylose was consumed. The amount of ethanol produced during glucose–xylose cofermentation increased with increasing initial cell concentrations (0 to 9 h; Fig. 3c) but showed no significant difference after 24 h of fermentation. As in the case for xylose fermentation, the maximum rate of ethanol production was enhanced by increasing the initial cell concentration, while the maximum ethanol concentration was almost the same for all concentrations (Table 2). Thus, MA-R4 could consume a high amount of substrate (glucose and xylose), thereby producing a high amount of ethanol at high initial cell concentrations. Taken together, the substrate consumption and ethanol production rates appear to largely depend on the initial cell concentration, and starting with a highly concentrated inoculum may be a promising strategy for improving the rate of substrate utilization and ethanol production.

Figure 4 shows that, by increasing initial cell concentrations (from 1.60 to 12.7 g/L), the maximum production of xylitol, glycerol, and acetic acid was increased from 3.71 to 4.44 g/L, 3.84 to 4.58 g/L, and 1.31 to 1.75 g/L, respectively. In contrast to xylose fermentation, the concentrations of all three by-products were slightly enhanced with increasing initial cell concentrations. Moreover, the xylitol yield was relatively constant except at the initial cell concentration of 12.7 g/L, while the glycerol and acetic acid yields slightly increased with increasing cell concentrations (Table 2). The ethanol yield from total consumed sugars (glucose and xylose) was 0.40, 0.40, 0.40, 0.39, and 0.38 g/L for initial cell concentrations of 1.60, 3.27, 6.76, 9.87, and 12.7 g/L, respectively. The average

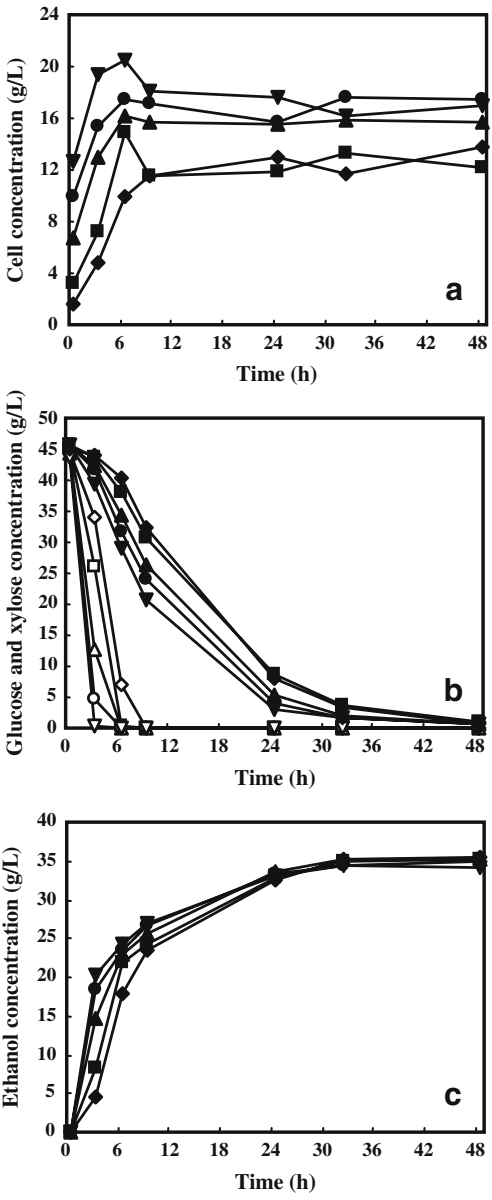

Fig. 3 Time-dependent ethanol fermentation profiles of **a** cell, **b** glucose (*open symbol*) and xylose (*closed symbol*), and **c** ethanol concentrations at different initial cell concentrations of recombinant *S. cerevisiae* strain MA-R4 in YPDX medium containing both glucose (45 g/L) and xylose (45 g/L). ♦, 1.60 g/L; ■, 3.27 g/L; ▲, 6.76 g/L; ●, 9.87 g/L; ▼, 12.7 g/L initial cells. Values are averages from three independent experiments

ethanol yield from cofermentation (0.39 g/g; 76.7% of the theoretical yield) was higher than that from xylose fermentation (0.34 g/g; 65.7% of the theoretical yield). It should be noted that higher yields of ethanol in the presence of glucose were also observed in other recombinant *S. cerevisiae* strains carrying the same genes on the chromosomes [8, 17], suggesting that the presence of glucose improved the ethanol production. For cofermentation, the ethanol yield remained unaffected by the initial cell concentration over the range of 1.60–6.76 g/L as with the fermentation from xylose but was slightly lower at higher initial cell concentrations (9.87 and 12.7 g/L). This lower ethanol yield at high initial cell

Table 2 Summary of 48-h fermentation of mixed sugars containing glucose and xylose by *S. cerevisiae* strain MA-R4.

Parameter	Initial cell concentration (g/L)				
	1.60±0.17	3.27±0.22	6.76±0.70	9.87±0.88	12.7±1.31
Initial glucose concentration (g/L)	44.7±1.0	45.1±0.7	44.5±1.9	44.3±0.8	43.8±0.3
Initial xylose concentration (g/L)	45.1±0.5	45.7±1.0	45.6±1.5	45.7±0.4	45.8±0.4
Maximum ethanol concentration (g/L)	35.5±0.35	35.5±0.16	35.4±0.32	34.9±0.44	34.5±0.96
Maximum glucose consumption rate (g/L h)	4.473±0.110	4.970±0.123	10.65±0.726	13.23±0.331	14.43±0.110
Maximum xylose consumption rate (g/L h)	1.541±0.101	1.560±0.112	1.683±0.090	2.414±0.203	2.788±0.278
Maximum ethanol production rate (g/L h)	2.610±0.032	3.632±0.035	4.856±0.122	6.126±0.162	6.683±0.306
Ethanol yield (g/g)	0.397±0.014	0.398±0.011	0.396±0.020	0.387±0.011	0.383±0.004
Xylitol yield (g/g)	0.097±0.008	0.091±0.006	0.094±0.003	0.094±0.002	0.103±0.010
Glycerol yield (g/g)	0.045±0.003	0.047±0.003	0.049±0.006	0.049±0.005	0.052±0.005
Acetic acid yield (g/g)	0.013±0.001	0.015±0.002	0.016±0.001	0.017±0.002	0.017±0.003

Values are averages of three independent experiments ± standard deviation

concentrations may be directly related to high by-product yields. These results suggest that selectivity for ethanol is high at low initial cell concentrations and decreases with increasing initial cell concentrations.

Conclusions

The effect of the initial cell concentration of flocculent industrial *S. cerevisiae* strain MA-R4 on growth, substrate consumption, ethanol production, and by-product formation in

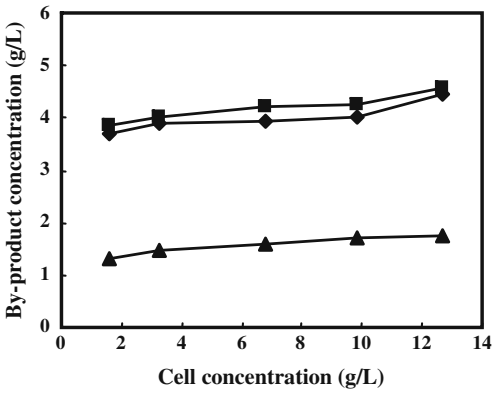

Fig. 4 Effect of initial cell concentration on the maximum production of xylitol (♦), glycerol (■), and acetic acid (▲) from glucose–xylose cofermentation by recombinant *S. cerevisiae* strain MA-R4. Values are averages from three independent experiments

complex medium containing either xylose or a mixture of glucose and xylose was investigated. Results showed that the initial cell concentration of MA-R4 affected the fermentation rate and product yields. The rate of substrate (glucose and xylose) consumption and ethanol production was high when the initial cell concentration was high, although MA-R4 was able to simultaneously coferment glucose and xylose to ethanol even at relatively low initial cell concentrations. When xylose alone was used as the fermentation substrate, the ethanol yield was constant even at high initial cell concentrations, while when both glucose and xylose were used it was slightly lower at high initial cell concentrations. These ethanol yields results are most likely due to the increased formation of by-products, including xylitol at a high initial cell concentration, supporting the idea that ethanol selectivity is reduced at higher initial cell concentrations. Based on these results, inoculation at the appropriate initial cell concentration is crucial for optimizing the design of the overall fermentation process to achieve complete substrate consumption using MA-R4. Further studies are undergoing to determine the effects of different initial cell concentrations on fermentation of lignocellulosic hydrolysates by MA-R4.

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