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Characterization of a Recombinant Flocculent *Saccharomyces cerevisiae* Strain that Co-ferments Glucose and Xylose: I. Influence of the Ratio of Glucose/Xylose on Ethanol Production

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Abstract Glucose/xylose mixtures (90 g/L total sugar) were evaluated for their effect on ethanol fermentation by a recombinant flocculent *Saccharomyces cerevisiae*, MA-R4. Glucose was utilized faster than xylose at any ratio of glucose/xylose, although MA-R4 can simultaneously co-ferment both sugars. A high percentage of glucose can increase cell biomass production and therefore increase the rate of glucose utilization (1.224 g glucose/g biomass/h maximum) and ethanol formation (0.493 g ethanol/g biomass/h maximum). However, the best ratio of glucose/xylose for the highest xylose consumption rate (0.209 g xylose/g biomass/h) was 2:3. Ethanol concentration and yield increased and by-product (xylitol, glycerol, and acetic acid) concentration decreased as the proportion of glucose increased. The maximum ethanol concentration was 41.6 and 21.9 g/L after 72 h of fermentation with 90 g/L glucose and 90 g/L xylose, respectively, while the ethanol yield was 0.454 and 0.335 g/g in 90 g/L glucose and 90 g/L xylose media, respectively. High ethanol yield when a high percentage of glucose is available is likely due to decreased production of by-products, such as glycerol and acetic acid. These results suggest that ethanol selectivity is increased when a higher proportion of glucose is available and reduced when a higher proportion of xylose is available.

Keywords Recombinant *Saccharomyces cerevisiae* · Xylose · Glucose · Ethanol · Co-fermentation · Ratio of sugar

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Introduction

The production of bioethanol for use as transport fuel has attracted much attention recently due to the need to reduce greenhouse gas emissions. Lignocellulosic biomass, such as agricultural and forest residues, is composed predominantly of cellulose, hemicelluloses, and lignin, and has been investigated as a source of potentially fermentable sugars present in the cellulose and hemicelluloses fractions. Cellulose is a linear polysaccharide polymer composed of many glucose monosaccharide units, while hemicellulose is a highly branched polymer made up of the hexose sugars glucose, mannose, and galactose, and the pentose sugars xylose and arabinose [1]. Xylose, the second most abundant monosaccharide in nature after glucose, is the predominant pentose sugar in hemicellulose derived from hardwoods and crop residues [2]. Therefore, the utilization of xylose is required to make lignocellulosic ethanol cost-competitive. This recognition has resulted in significant effort by numerous researchers to engineer organisms and their pathways to convert xylose into ethanol [3, 4].

The yeast *Saccharomyces cerevisiae* is one of the most promising candidates for the industrial-scale production of ethanol owing to its high ethanol yield and productivity from hexose sugars. Furthermore, *S. cerevisiae* is more robust than bacteria and other yeasts with regard to tolerance to ethanol and lignocellulose-derived inhibitors [5]. However, *S. cerevisiae* cannot utilize xylose for growth or fermentation due to its lack of an active catabolic pathway for this sugar. On the other hand, xylulose, a keto-isomer of xylose, can be phosphorylated to xylulose 5-phosphate and degraded via the pentose phosphate pathway in *S. cerevisiae*. Although many *S. cerevisiae* strains capable of utilizing xylose for ethanol production have been engineered [6–10], processes utilizing these strains cannot economically produce ethanol from lignocellulosic biomass, mainly because these strains cannot yet efficiently convert xylose to ethanol with the high yields and fermentation rates possible with glucose. We previously reported the development of a recombinant industrial *S. cerevisiae* strain, MA-R4, that can simultaneously co-ferment glucose and xylose to ethanol and that has high ethanol productivity [11]. The MA-R4 strain was engineered by chromosomal integration to express the *XYL1* and *XYL2* genes that encode XR and XDH from *Scheffersomyces* (*Pichia*) *stipitis*, along with the *S. cerevisiae XKS1* gene that encodes XK using the alcohol-fermenting flocculent yeast strain IR-2. IR-2 has the best performance in xylulose-to-ethanol conversion among many different industrial *S. cerevisiae* strains, implying that IR-2 has a promising genetic background that could be beneficial to anaerobic xylose fermentation [12].

Optimization of fermentation conditions and environmental parameters, such as the composition of the culture medium, are important for bioconversion productivity [13], and for obtaining both the maximum ethanol production rate and maximum ethanol yield from xylose [14]. For instance, the initial cell concentration of the MA-R4 strain greatly affects the rate of xylose fermentation and ethanol production [15]. To increase the scale of the overall design of processes for production of ethanol from lignocellulosic biomass, it is thus necessary to understand the ethanol production kinetics in single sugar and mixed-sugar fermentation. Glucose and xylose are the predominant sugars in hardwood and grass hemicelluloses, and in practice, hydrolysates of lignocellulosic biomass have different ratios of glucose/xylose, depending on the types of substrate and pretreatment. However, few studies into fermentation of glucose and xylose mixtures by engineered *S. cerevisiae* strains with xylose-fermenting ability have been reported. Therefore, the aim of this study was to examine the effects of various glucose/xylose ratios on cell growth, substrate utilization, ethanol concentration, and ethanol and by-product yield using MA-R4.

Materials and Methods

Microorganisms and Media

The xylose-fermenting recombinant *S. cerevisiae* strain MA-R4, derived from the diploid and flocculent yeast strain IR-2 [16], was used in this study. MA-R4 was genetically engineered with the chromosome-integrated *XYL1* and *XYL2* genes that encode XR and XDH from *S. stipitis*, along with the endogenous *XKS1* gene that encodes XK under the control of the *PGK* promoter [11]. Briefly, plasmid pAUR-XKXDHXR [17] was digested with the restriction enzyme *Bsi*WI and 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). Glucose (90 g/L) was added to YP medium to produce YP9D medium. The addition of xylose (90 g/L) to YP medium produced YP9X medium. For glucose/xylose mixtures, different masses of glucose and xylose were added to YP medium to produce a final total sugars concentration of 90 g/L. Four mixtures with different glucose/xylose ratios (4:1, 3:2, 2:3 and 1:4, *w*/*v* ratios) in complex medium were prepared to produce YP4D1X, YP3D2X, YP2D3X, and YP1D4X media, respectively. These glucose/xylose ratios were selected based on a previous study [18] using another xylose-utilizing *S. cerevisiae* strain. The four media (YP4D1X, YP3D2X, YP2D3X, and YP1D4X), together with YP9D and YP9X, were used as fermentation media in the present study. The compositions of these media are shown in Table 1.

Fermentation

For anaerobic batch fermentation, MA-R4 was first cultivated aerobically in 5 mL of YPD medium for 36 h at 30 °C. Then, the culture was centrifuged at 5,000 rpm for 5 min at 4 °C, and the pelleted cells were washed and resuspended in distilled water. These cells were inoculated into 20 mL of fermentation medium (YP9D, YP4D1X, YP3D2X, YP2D3X, YP1D4X, and YP9X). For all fermentation media, the initial cell density was adjusted to approximately 4.01 g (dry cell weight (DCW))/L. Anaerobic batch fermentations were performed at 30 °C in sterile closed bottles (50 mL) with magnetic stirring, as described previously [11, 12, 15]. Samples (0.3 mL) of fermentation broth (YP9D, YP4D1X, YP3D2X, YP2D3X, YP1D4X, and YP9X) were taken at specified intervals and diluted ten times with 8 mM H_2SO_4. These diluted samples were stored at −30 °C for high-

Table 1 Composition of different media used for 72-h fermentation by *S. cerevisiae* strain MA-R4

Medium	Glucose concentration[a] (g/L)	Xylose concentration[a] (g/L)	Total sugar concentration[a] (g/L)	Glucose/xylose ratio
YP9D	90.4±2.4	0	90.4±2.4	NA
YP4D1X	72.1±1.4	18.1±0.2	90.2±1.5	4:1
YP3D2X	54.7±0.3	36.8±0.5	91.5±0.5	3:2
YP2D3X	34.5±1.7	53.4±0.8	88.6±2.3	2:3
YP1D4X	17.7±1.0	71.3±0.9	89.0±1.8	1:4
YP9X	0	89.3±1.6	89.3±1.6	NA

Values are means of three independent experiments ± standard deviation*NA* not applicable

performance liquid chromatography (HPLC) analysis of substrates and fermentation products. All experiments were performed in triplicate.

Analytical Methods

DCW was determined using a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan) to measure the absorbance of the samples at 600 nm, as described previously [17]. Concentrations of glucose, xylose, ethanol, xylitol, glycerol, and acetic acid were determined with an HPLC apparatus (Jasco, Tokyo, Japan) equipped with a refractive index detector (RI-2031Plus; Jasco) using an Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA) 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

Sugar Consumption

Figure 1 shows the ethanol fermentation profiles of MA-R4 in YP media supplemented with different concentrations of glucose and xylose (90 g/L total sugar). Several fermentation parameters are shown in Table 2. MA-R4 rapidly fermented and depleted glucose within 9 h when glucose was the sole carbon source (Fig. 1a). However, when xylose was the sole carbon source, MA-R4 converted xylose very slowly (Fig. 1f), consuming only 73 % of the xylose after 72 h. In mixed-sugar fermentation media containing both glucose and xylose in the ratios 4:1, 3:2, 2:3, and 1:4 (Table 1), MA-R4 was able to simultaneously co-ferment glucose and xylose (0 to 9 h; Fig. 1b, 0 to 6 h; Fig. 1c, 0 to 6 h; Fig. 1d, 0 to 3 h; Fig. 1e), although glucose was utilized more rapidly than xylose. The co-fermentation performance of MA-R4 is consistent with our previous findings [11, 15]. After the glucose was completely consumed, almost all the xylose was metabolized within 72 h (Fig. 1b–e). The amount of consumed glucose per unit time during glucose fermentation increased with the percentage of glucose in the fermentation medium. Meanwhile, after the glucose was completely metabolized, the amount of consumed xylose per unit time increased with proportion of xylose.

During this experiment, the average glucose consumption rate (0.967 g glucose/g DCW/h) was much higher than the average xylose consumption rate (0.154 g xylose/g DCW/h). The maximum specific glucose consumption rate was enhanced by increasing the initial glucose concentration (Table 2). On the other hand, the maximum specific xylose consumption rate by MA-R4 increased from 0.072 to 0.209 g xylose/g DCW/h during fermentation of mixed sugars in which the ratios of glucose and xylose varied from 4:1 to 2:3 (YP4D1X, YP3D2X and YP2D3X media). Surprisingly, however, the maximum specific xylose consumption rate by MA-R4 was slightly lower in YP1D4X (1:4 glucose/xylose) and YP9X (xylose alone) media when compared with YP2D3X medium (Table 2). The specific xylose consumption rate (0.072 g xylose/g DCW/h) of MA-R4 during fermentation in YP4D1X was lower by almost one-third the rate (0.209 g xylose/g DCW/h) in YP2D3X. Thus, the specific glucose consumption and ethanol production rates of MA-R4 largely depend on the proportion of glucose present in the fermentation medium, but the specific xylose consumption rate by MA-R4 appears to be the highest when almost equivalent amounts of glucose and xylose are present. In this study, the best ratio of glucose to xylose for a high specific xylose consumption rate by MA-R4 is 2:3. These findings are not consistent with those obtained using other xylose-utilizing yeast; it has

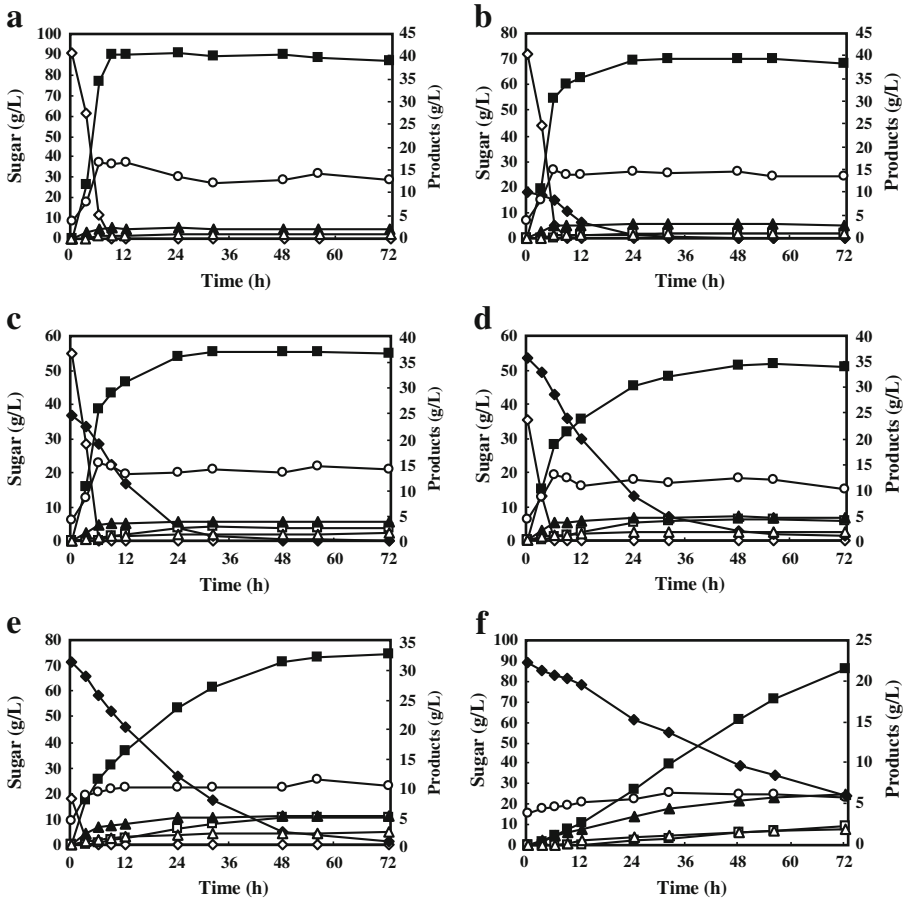

Fig. 1 Time-dependent ethanol fermentation profiles of recombinant *S. cerevisiae* strain MA-R4 in **a** YP9D, **b** YP4D1X, **c** YP3D2X, **d** YP2D3X, **e** YP1D4X and **f** YP9X media. *open diamond* Glucose, *filled diamond* xylose, *filled square* ethanol, *open square* xylitol, *filled upright triangle* glycerol, *open upright triangle* acetic acid, *open circle* dry cell weight. Values are averages from three independent experiments

been reported that the xylose consumption rate increased as the proportion of glucose was reduced [19, 20]. Currently, it is difficult to fully account for these differences in xylose consumption rates, but it may be yeast strain-dependent. It is also possible that competition for unspecific hexose transporters mediating uptake of xylose in MA-R4 may influence the rate of xylose consumption [21]. In addition, the strength of the promoter in the genetic constructs may be affected by the presence or absence of glucose, although the promoters used are generally considered to be constitutive. Regardless, glucose may be involved in an as yet unknown mechanism underlying the rate of xylose consumption by this strain.

Cell Growth

In the fermentation experiments, MA-R4 grew quickly during glucose fermentation using YP9D (100 % glucose) medium (Fig. 1a), but scarcely grew at all during xylose fermentation using YP9X (100 % xylose) medium (Fig. 1f). During fermentation using glucose and

Table 2 Summary of 72-h fermentation of single and mixed sugar media by *S. cerevisiae* strain MA-R4

Parameter	Medium					
	YP9D	YP4D1X	YP3D2X	YP2D3X	YP1D4X	YP9X
Maximum cell concentration (g/L)	19.08±1.16	16.81±0.77	15.96±1.17	13.90±0.51	11.04±0.70	6.50±0.50
Maximum ethanol concentration (g/L)	41.6±0.76	40.2±0.49	38.0±0.41	34.9±1.33	33.0±0.34	21.9±1.22
Maximum specific glucose consumption rate (g glucose/g DCW/h)	1.224±0.138	1.115±0.095	1.022±0.103	0.867±0.039	0.606±0.024	ND
Maximum specific xylose consumption rate (g xylose/g DCW/h)	ND	0.072±0.014	0.131±0.023	0.209±0.033	0.189±0.010	0.168±0.011
Maximum specific ethanol production rate (g ethanol/g DCW/h)	0.493±0.084	0.423±0.071	0.413±0.047	0.388±0.033	0.300±0.014	0.051±0.002
Biomass yield (g/g)	0.183±0.022	0.164±0.008	0.159±0.006	0.140±0.025	0.130±0.005	0.113±0.002
Ethanol yield (g/g)	0.454±0.009	0.448±0.009	0.418±0.003	0.406±0.011	0.384±0.006	0.335±0.003
Xylitol yield (g/g)	ND	0.069±0.005	0.075±0.004	0.081±0.003	0.069±0.002	0.036±0.003
Glycerol yield (g/g)	0.028±0.004	0.034±0.003	0.042±0.002	0.053±0.003	0.058±0.001	0.094±0.007
Acetic acid yield (g/g)	0.008±0.001	0.009±0.000	0.013±0.001	0.018±0.001	0.023±0.001	0.029±0.004

Values are means of three independent experiments ± standard deviation

ND not detectable

xylose mixed substrates, cell biomass increased quickly in the co-consumption phase of glucose and xylose (0 to 9 h; Fig. 1b, 0 to 6 h; Fig. 1c, 0 to 6 h; Fig. 1d, 0 to 3 h; Fig. 1e), and then increased slowly or scarcely at all in the xylose-only consumption phase (9 to 72 h; Fig. 1b, 6 to 72 h; Fig. 1c, 6 to 72 h; Fig. 1d, 3 to 72 h; Fig. 1e). Thus, like many other yeasts, MA-R4 preferentially utilizes glucose. An increase in the initial glucose concentration in the media resulted in an increase in the maximum cell concentration (Table 2). The highest cell concentration (19.08 g/L) was achieved from fermentation in the presence of glucose as the sole carbon source, and the lowest cell concentration (6.50 g/L) was achieved from fermentation in the presence of xylose alone. This result is in agreement with studies by Agbogbo et al. [19], in which the highest and the lowest final cell concentration of *P. stipitis* at the end of fermentation were in 100 % glucose and 100 % xylose media, respectively. Thus, MA-R4 grew faster on glucose as the sole carbon source than on xylose alone. Furthermore, the biomass yield per gram of total consumed sugars (g/g) by MA-R4 also increased with the proportion of glucose (Table 2). Cell growth was rapid when a high percentage of glucose was available, which leads to a high rate of glucose consumption and ethanol production. In contrast, the xylose consumption rate is determined by a more complex set of factors, as discussed in the previous section.

Ethanol Production

As expected, ethanol production paralleled substrate utilization, as evidenced by the ethanol concentration data shown in Fig. 1. In YP9D medium containing 100 % glucose, MA-R4 rapidly produced 41.6 g/L ethanol after 9 h of fermentation (Fig. 1a), whereas MA-R4 gradually produced 21.9 g/L ethanol after 72 h in YP9X medium containing 100 % xylose (Fig. 1f). In fermentations using mixtures of glucose and xylose, ethanol was quickly produced during the glucose/xylose co-consumption phase (0 to 9 h; Fig. 1b, 0 to 6 h; Fig. 1c, 0 to 6 h; Fig. 1d, 0 to 3 h; Fig. 1e), whereas ethanol was gradually produced during the xylose consumption phase (9 to 72 h; Fig. 1b, 6 to 72 h; Fig. 1c, 6 to 72 h; Fig. 1d, 3 to 72 h; Fig. 1e). The maximum ethanol concentration increased with the percentage of glucose (Table 2 and Fig. 2), or, put another way, the proportion of xylose relative to glucose

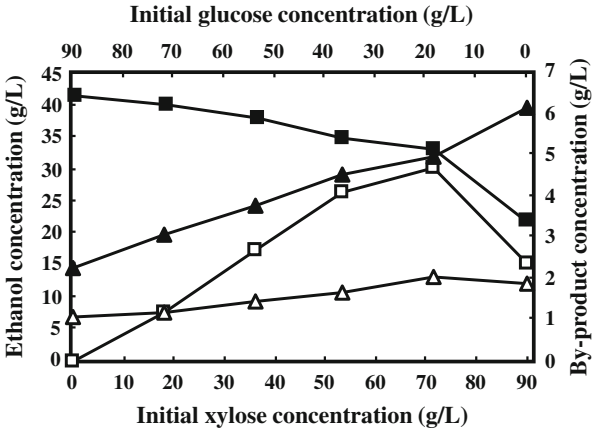

Fig. 2 Effects of initial glucose/xylose ratio on the maximum production of ethanol (*filled square*) and by-products (xylitol (*open square*), glycerol (*filled upright triangle*) and acetic acid (*open upright triangle*)) from fermentation of single and mixed sugar media by recombinant *S. cerevisiae* strain MA-R4. Values are averages from three independent experiments

increased with decreasing ethanol concentration (Fig. 2). The highest ethanol concentration (41.6 g/L) was achieved in fermentation with glucose as the sole carbon source, and the lowest ethanol concentration (21.9 g/L) was achieved in fermentation with xylose alone. At any point in time, the amount of produced ethanol was high when the percentage of available glucose was high.

As with sugar utilization, the maximum specific ethanol production rates increased with the proportion of glucose (Table 2). The highest rates of specific ethanol production (0.493 g ethanol/g DCW/h) were achieved in fermentation with glucose alone (Table 2). In contrast, MA-R4 exhibited a very slow maximum specific ethanol production rate (0.051 g ethanol/g DCW/h) in fermentation with xylose alone, in which the rate of specific ethanol production in YP9X was lower, at almost one-tenth the rate in YP9D (Table 2). The ethanol yield per gram of total consumed sugars (grams per gram) of MA-R4 was also affected by the ratio of glucose/xylose, with the ethanol yield increasing with the percentage of glucose (Table 2). Consequently, the ethanol yield from glucose fermentation (0.454 g/g) was higher than that from xylose (0.335 g/g). It should be noted that higher ethanol production rates and higher ethanol yields as the proportion of glucose increased were also observed in another xylose-utilizing yeast, *Pachysolen tannophilus* [20], and the previously reported recombinant strains of laboratory xylose-utilizing *S. cerevisiae*, MA-N4 [17] and MA-B43 [22] (data not shown), thus suggesting that an increase in the proportion of glucose improved the production rate and yield of ethanol by these yeast strains. However, MA-N4 and MA-B43 exhibited approximately twofold-lower specific ethanol production rates than MA-R4 (data not shown), thus supporting the notion that MA-R4 is the predominant yeast strain responsible for the efficient production of ethanol. Thus, ethanol yield, ethanol concentration and rate of ethanol production by MA-R4 during fermentation appear to depend on the ratio of glucose/xylose in the fermentation medium.

By-Product Production

Fermentation of YP9D medium containing 90 g/L glucose alone resulted in no production of xylitol (Fig. 1a). Xylitol production was maintained below 4.70 g/L during fermentation on glucose and xylose mixed substrates (Fig. 1b–e) and on xylose alone (Fig. 1f). For all media, a small amount of glycerol (no more than 6.13 g/L) and a small amount of acetic acid (no more than 2.06 g/L) was produced, mainly during the glucose consumption phase (Fig. 1). Consequently, as shown in Fig. 2, by increasing the concentration of xylose (from 0 to 71.3 g/L), the maximum production of xylitol and acetic acid was increased from 0 to 4.70 g/L and 1.07 to 2.06 g/L, respectively, but was reduced from 4.70 to 2.35 g/L and 2.06 to 1.89 g/L, respectively, when xylose was the sole carbon source. The decrease in formation of xylitol and acetic acid in the 100 % xylose medium is most likely due to the fact that the amount of xylose available, approximately 90 g/L, was not completely consumed after 72 h of fermentation (Fig. 1f). On the other hand, the maximum production of glycerol was enhanced from 2.27 to 6.13 g/L by increasing the xylose concentration (from 0 to 89.3 g/L). While the glycerol and acetic acid yield decreased linearly as the percentage of glucose increased, the xylitol yield was highest in fermentation on mixed sugars in which the proportion of glucose/xylose was 2:3 (YP2D3X medium). The lower glycerol and acetic acid yield at high proportions of glucose may be directly related to high ethanol yields. These results suggest that selectivity for ethanol is high at high proportions of glucose, and decreases as the proportion of xylose increases.

Conclusions

Glucose and xylose are generally the predominant sugars in lignocellulosic hydrolysates, although their relative ratios depend on the pretreatment and hydrolysis technology used. We therefore investigated the effects of various glucose/xylose mixtures, with an initial total sugar concentration of 90 g/L in complex medium, on growth, substrate consumption, ethanol production and by-product formation. The flocculent industrial *S. cerevisiae* strain MA-R4 was used, and all media were inoculated with the same initial cell concentration. The results showed that different proportions of glucose to xylose in fermentation media affect various fermentation parameters. When a high percentage of glucose was available, cell growth was rapid, leading to a high rate of glucose consumption and ethanol production. In contrast, the xylose consumption rate appeared to be independent of both the initial xylose concentration and of cell growth. Ethanol concentration and yield increased with the proportion of glucose. High ethanol yield appeared to be related to decreased formation of by-products, including glycerol and acetic acid, in the presence of a high percentage of glucose, supporting the notion that ethanol selectivity is reduced when a higher percentage of xylose is present. No xylitol formation was observed in fermentation of glucose alone, and the ratio of glucose to xylose for the highest yield of xylitol was 2:3. From these results, we conclude that control of extracellular glucose concentration is important for effective glucose/xylose co-fermentation. Further studies have been performed to optimize fermentation conditions for ethanol production using MA-R4, and the results are presented elsewhere [23].

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