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Adaptation Yields a Highly Efficient Xylose-Fermenting *Zymomonas mobilis* Strain

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ABSTRACT: *Zymomonas mobilis* is a superb ethanol producer with productivity exceeding yeast strains by several fold. Although metabolic engineering was successfully applied to expand its substrate range to include xylose, xylose fermentation lagged far behind glucose. In addition, xylose fermentation was often incomplete when its initial concentration was higher than 5%. Improvement of xylose fermentation is therefore necessary. In this work, we applied adaptation to improve xylose fermentation in metabolically engineered strains. As a result of adaptation over 80 days and 30 serial transfers in a medium containing high concentration of xylose, a strain, referred as A3, with markedly improved xylose metabolism was obtained. The strain was able to grow on 10% (w/v) xylose and rapidly ferment xylose to ethanol within 2 days and retained high ethanol yield. Similarly, in mixed glucose–xylose fermentation, a total of 9% (w/v) ethanol was obtained from two doses of 5% glucose and 5% xylose (or a total of 10% glucose and 10% xylose). Further investigation reveals evidence for an altered xylitol metabolism in A3 with reduced xylitol formation. Additionally xylitol tolerance in A3 was increased. Furthermore, xylose isomerase activity was increased by several times in A3, allowing cells to channel more xylose to ethanol than to xylitol. Taken together, these results strongly suggest that altered xylitol metabolism is key to improved xylose metabolism in adapted A3 strain. This work further demonstrates that adaptation and metabolic engineering can be used synergistically for strain improvement.

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KEYWORDS: *Zymomonas mobilis*; xylose fermentation; ethanol; xylitol; adaptation

Introduction

Lignocellulose is the most abundant source of renewable carbon in the biosphere (Claassen et al., 1999). Synthesized at a scale of 10^{11} tons annually, it has an energy content equivalent to 640 billion tons of crude oils (Proctor et al., 2005). Utilizing this vast renewable resource for energy production requires innovative technologies. Although lignocellulosic ethanol has long been considered as a viable replacement for gasoline as transportation fuel, its large scale production is still hampered by the high production cost. *Zymomonas mobilis* is one of the best ethanol producers found in nature. Its high ethanol yield (97% of the theoretic value), higher productivity than the common yeast strain, make it particularly attractive as biocatalyst in ethanol fermentation (Panesar et al., 2006; Rogers et al., 1982; Swings and De Ley, 1977). However, these values only pertain to its ability to use glucose. *Z. mobilis* naturally does not ferment xylose. An important breakthrough came when Zhang et al. (1995) successfully applied metabolic engineering to expand its substrate range to xylose. In the ensuing decade, however, xylose metabolism continued to be a focal point of research as it was found that the ability of engineered strains to metabolize xylose was considerably lower than that of glucose and improvement was needed (De Graaf et al., 1999; Gao et al., 2002; Jeon et al., 2005; Kim et al., 2000).

The most significant difficulties with xylose fermentation by engineered *Z. mobilis* were lower xylose consumption rate, which led to a long fermentation time and incomplete xylose utilization when xylose concentration was high. Much research over the past decade has been devoted to identify potential bottlenecks in xylose fermentation. Studies using NMR techniques suggested that xylose-metabolizing cells were in a less energized state compared to glucose-metabolizing cells (Kim et al., 2000). A 1992 study by Feldmann et al. (1992) was the first to identify that xylitol formed during xylose metabolism was a significant limiting factor. Besides detracting from ethanol yield, xylitol was further converted to xylitol phosphate, a potent growth

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inhibitor, by heterologous xylulokinase, a necessary enzyme in xylose metabolism (Akinterinwa and Cirino, 2009; Feldmann et al., 1992). Additionally, xylitol was shown as a competitive inhibitor for xylose isomerase isolated from an *Arthrobacter* strain with K_i 0.3 mM (Smith et al., 1991). This was likely to be true for xylose isomerase from *Escherichia coli* (Viitanen et al., 2008). Suppressing xylitol formation, therefore, could lead to improved xylose metabolism. Indeed, as described in a US patent (Viitanen et al., 2008), a strain ZW800 with reduced xylitol formation was generated by knocking out the gene for glucose-fructose oxidoreductase (GFOR), removing one of the mechanisms responsible for xylitol synthesis. The mutant showed significantly improved xylose metabolism, compared to its parental strain. An added advantage for the mutant strain was the ability to tolerate acetate. For example, in a mixed sugar fermentation with 92 g/L glucose and 97 g/L xylose, in the presence of 7.2 g/L acetate and 10 mM sorbitol, a majority of xylose was consumed and xylose concentration was reduced to about 30 g/L over a fermentation period of 50 h, producing about 72.3 g/L ethanol (Viitanen et al., 2008).

In this work, we used an alternative approach, adaptation or adaptive mutation, to improve xylose utilization in a rationally engineered strain. This evolutionary approach has been proven in recent literature as a powerful tool for strain improvement, especially combined with rational metabolic engineering (Fong et al., 2003; Kuyper et al., 2005; Lawford et al., 1998, 1999; Meijnen et al., 2008; Rosenberg, 2001; Viitanen et al., 2008; Zhang et al., 2002). For example, an adapted recombinant *Zymomonas* strain was obtained using continuous culture in a medium containing hardwood hydrolysate (Lawford et al., 1999). In a more recent study, xylose fermentation was significantly improved in an adaptation involving about 40 transfers for *Pseudomonas putida* (Meijnen et al., 2008). This approach is easy to implement and widely applicable as it requires no detailed knowledge of the bottlenecks of the target metabolism. The latter aspect is relevant to xylose fermentation in *Z. mobilis* as even in improved strains, xylose fermentation still lags behind glucose and the reasons for this are not completely clear. A good example for the incomplete picture of xylose fermentation in this organism is mechanism for xylitol formation. Besides GFOR, a NADPH-dependent xylose reductase was the second enzyme responsible for xylitol formation (Feldmann et al., 1992; Viitanen et al., 2008), yet this enzyme remains elusive. While an adaptation is relatively easy to set up, the specific conditions need to be selected carefully in order to yield strains with desired traits. As previously metabolically engineered strains appeared to have difficulties in dealing with high concentration of xylose, we chose 5% xylose as the sole carbon source in the adaptation experiment. This condition was not previously used in adaptation for *Zymomonas*.

We present here a comparative study of two adapted strains, referred as A1 and A3, respectively. A1 was obtained by transforming ZM4 (ATCC 31821) with an expression vector carrying four *E. coli* xylose metabolic genes (Fig. 1), followed by a brief adaptation involving only 4 transfers. This brief adaptation was necessary in order to obtain a clone that grew on xylose as sole carbon source. The need for an initial adaptation was reported by others (Viitanen et al., 2008; Zhang et al., 2002). To further improve xylose fermentation, an extended adaptation involving 30 transfers was carried out for about 80 days, resulting in a markedly improved strain, designated as A3. We show that A3 outperforms A1 in xylose consumption rate by several fold, and compares favorably with other reported *Zymomonas* strains in the ability to ferment high concentration of xylose, demonstrating the power of using extended adaptation in improving engineered strains. Further analysis of the two strains indicates altered xylitol metabolism is, at least partially, responsible for the improvement.

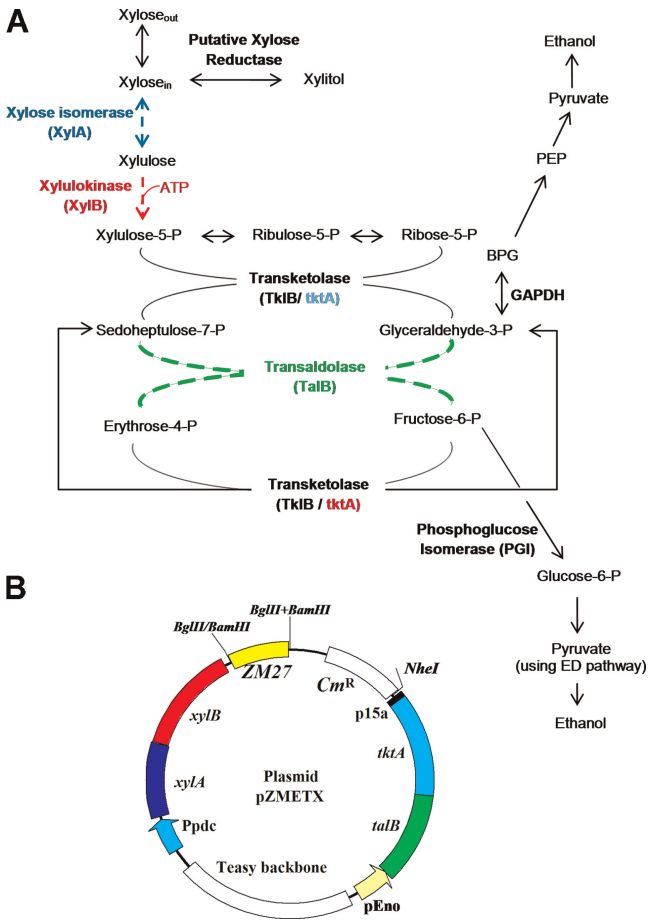

Figure 1. **A**: Xylose metabolizing pathway in engineered *Z. mobilis*. Native enzymes (genes) are shown in black. Heterologous enzymes (genes) are shown in other colors. Engineered *Z. mobilis* has two transketolase genes—native tklB and heterologous tktA. **B**: Plasmid map for pZMETX. P, phosphate; BPG, 1,3-bisphosphoglycerate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ppdc, promoter for pyruvate decarboxylase; Peno, promoter for enolase; xylA, xylose isomerase; xylB, xylulokinase; talB, transaldolase; tklB/tktA, transketolase; CmR, chloramphenicol resistance gene; ZM27, "maintain region" of a native plasmid; p15a, *E. coli* origin of replication. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

Materials and Methods

Organisms and Media

Zymomonas mobilis ZM4 transformants were grown in RM medium (1% yeast extract, 0.2% KH_2PO_4) supplemented with 100 μg/mL chloramphenicol and different amounts of glucose or xylose (as indicated) as carbon source. *E. coli* strains (W3110 and JM109) were grown in Luria–Bertani (LB) medium supplemented with 100 μg/mL ampicillin. Agar plates contained the same media except with additional 1.5% agar.

Culture Conditions

E. coli cells were grown at 37°C in culture tube or shake flask on a biological shaker shaken at 250 rpm. *Z. mobilis* was grown at 30°C. Pre-seed culture (PSC) and seed culture (SC) of *Z. mobilis* were cultivated in 15-mL centrifuge tube and 100-mL Pyrex screw-cap bottle, respectively, filled to 60% volume and shaken at 250 rpm. PSC was prepared by inoculating a single colony from agar plate into the liquid medium. For 5% glucose–5% xylose fermentation, PSC contained 2.5% glucose–2.5% xylose while for 10% xylose fermentation, PSC contained 5% xylose. PSC was grown to the stationary phase. SC was prepared by inoculating it to an optical density (OD) of 0.1 using the stationary phase PSC. SC contained the same amount of sugars as the fermentation media in bioreactors. Appropriate amount of cells were harvested from SC at exponential phase, resuspended in fresh medium and were used to inoculate the bioreactor to get a starting OD of 0.1. The fermenter used (Infors HT Multifors, Bottmingen, Switzerland) had 4 vessels each with a working volume of 500 mL. The fermenter was stirred at 300 rpm. Condensers for the fermenter were kept at 1°C using a chiller. Before the inoculation, fermenter was purged with N_2 gas at 0.2 lpm and pH was adjusted to 5.75 by addition of 1.7 M KOH and 1 M H_3PO_4. During fermentation, acid was not used for pH control. However, it was observed that the pH in the vessel was in a narrow range of 5.75–6.0. To maintain anaerobic conditions, the exhaust tube was immersed in a water column to prevent atmospheric oxygen from diffusing into the fermenter vessels. Dissolved oxygen was monitored and was found to remain close to 0% throughout the fermentation. There was negligible loss of ethanol due to evaporation in this set up. Three replicates were done for each experiment starting from three colonies on an agar plate.

Analytical Methods

Cell growth was determined by measuring optical density (OD) at 600 nm. One OD_{600} on our spectrophotometer (Beckman Coulter DU 530, Brea, CA, Coulter DU 530) corresponds to 0.4 mg dry cell weight (DCW) of *Z. mobilis* per mL culture broth. Samples taken during fermentation were spun down by centrifugation and supernatants were analyzed for concentrations of glucose, xylose, lactic acid, glycerol and ethanol using high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA) instrument equipped with Supelcogel column H. All concentrations in units of % should be read as % (w/v) unless otherwise mentioned. Overlapping between xylose and xylitol peak was sometimes observed. Xylitol concentration was determined enzymatically. The reaction was carried out in 42 mM Tris–HCl buffer at pH 8.5, 5 mM NAD, 10 mM $MgSO_4$, 4 U/mL sorbitol dehydrogenase (SDH) and 10 μL of xylitol containing sample. Total amount of NADH produced by xylitol containing samples were compared to that produced by xylitol standards for xylitol estimation.

Enzymatic Assays

Sample preparations were according to a published procedure (Akinterinwa and Cirino, 2009). Briefly cells were harvested by centrifugation at 3,000*g* at 4°C for 15 min. Cells were washed once with extraction buffer [10 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 1 mM dithothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Washed cell pellets were stored at −20°C until use. Cell pellets were resuspended to an OD_{600} of 50 in the extraction buffer and sonicated (8 cycles of 10 s with 30 s cooling period). Cell debris was discarded after centrifugation at 53,300*g* for 20 min at 4°C. Supernatant was used as cell-free extract for enzymatic assays. Bradford Assay was used for estimation of protein concentration in the extract.

All enzymatic assay reactions were carried out in a final volume of 200 μL on a microplate. NAD(P)H absorbance was measured at 340 nm using Spectramax M5 Plus spectrophotometer (Molecular Devices, Sunnyvale, CA). Unless otherwise indicated, all commercial enzymes required for enzymatic assays were purchased from Sigma–Aldrich (St. Louis, MO).

Xylose isomerase (XI) activity was assayed in a reaction mixture containing 1 mM triethanolamine (TEA) (pH 7.5), 0.3 mM NADH, 100 mM xylose, 10 mM $MgSO_4$, 4 U/mL SDH (Roche Diagnostics, Indianapolis, IN) and 10 μL of crude cell extract (Gao et al., 2002; Schellenberg et al., 1984). Xylulokinase was assayed by a method a described in Akinterinwa and Cirino (2009). A solution containing 87 mM TEA (pH 8.5), 17 mM EDTA, 4.2 mM fructose-6-phosphate (F6P), 0.3 mM NADH, 2.5 U/mL glycerol-3-phosphate dehydrogenase, 10 U/mL triosephosphate isomerase (TPI) and 10 μL crude extract was used for transaldolase assay. Reaction was started by adding 25 μL of 12 mM erythrose-4-phosphate. 42 mM Tris–HCl (pH 8.5), 5 mM $MgCl_2$, 1 mM thiamine pyrophosphate (TPP), 0.5 mM ribose-5-phosphate, 0.2 mM NADH, 2.5 U/mL glycerol-3-phosphate dehydrogenase, 10 U/mL TPI, and 10 μL crude extract were used for transketolase assay. Reaction was started by adding 20 μL of 5 mM xylulose-5-phophate. Phosphoglucose isomerase (PGI) was assayed in a 50 mM Tris–Cl buffer (at pH 8) containing 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 3 mM NAD,

2 mM F6P, 1 U/mL glucose-6-phosphate dehydrogenase and 10 μL crude extract. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assayed in a 42 mM Tris–Cl buffer (at pH 8.5) containing 10 mM trisodium phosphate, 1 mM NAD, 3 mM β-mercaptoethanol, 1 mM DL-glyceraldehyde-3-phosphate (GAP) and 10 μL crude extract. This method is similar to that described in Iddar et al. (2002). Putative xylose reductase was assayed in McIlvaine buffer at pH 7.2 (prepared by adding 16.5 mL of 0.2 M sodium phosphate dibasic to 3.5 mL of 0.1 M citric acid) containing 0.35 mM NADPH, 0.26 M xylose and 5 μL crude cell extract (Viikari and Korhola, 1986).

Statistical significance of the enzymatic assay data was determined using Microsoft excel's *t*-test: Two-sample assuming equal variance.

Cloning of *E. coli* Xylose Metabolic Genes Into *Z. mobilis*

A plasmid pZMETX was designed and constructed to express four *E. coli* xylose metabolic genes, xylA (xylose isomerase), xylB (xylulokinase), talB (transaldolase), and tktA (transketolase). The expression was driven by two *Z. mobilis* promoters (Fig. 1).

Promoter-less xylAB operon was cloned from *E. coli* K-12 substr. W3110 and fused to the 500 bp pyruvate decarboxylate promoter (Ppdc) cloned from *Z. mobilis* ZM4. talB and tktA (along with its ribosome binding site), were cloned from W3110 in separate steps and fused together after the 500bp enolase promoter (Peno) cloned from ZM4. The two constructed operons—Ppdc–xylA–xylB and Peno–talB–tktA—were then ligated separately into two pGem®Teasy vectors (Promega Corp., Madison, WI) and then subcloned into vector pBluescript KS (Stratagene, Santa Clara, CA) to obtain pETX. 1.7 kb maintenance region 'ZM27' was PCR amplified from ORF1 of 2.7 kb native plasmid pZMO3 of *Z. mobilis* ATCC 10988 (Afendra et al., 1999). ZM27 "maintain region" enables the plasmid to be maintained and replicated within *Z. mobilis* cells. ZM27 was then ligated into commercially available vector pSTV28 (Takara Bio, Shiga, Japan) which has a chloramphenicol resistance gene and p15A origin of replication to obtain pSTVZM27. pSTVZM27 was electroporated into ZM4 to obtain control strain ZM4/pSTVZM27 using *E. coli* JM109 as an intermediate host. ZM4/pSTVZM27 thus does not contain xylose metabolizing genes. pETX and pSTVZM27 were ligated together to obtain pZMETX (Fig. 1B). pZMETX was first transformed into *E. coli* JM109 to yield JM109/pZMETX. Plasmid extracted from JM109/pZMETX was then electroporated into *Z. mobilis* ZM4 to obtain ZM4/pZMETX.

RNA Extraction and RT-PCR

RNA extraction was done using RNeasy Protect Bacteria Mini Kit (Qiagen Inc., Valencia, CA). Extracted RNA was treated with Ambion® TURBO DNA-*free*™ to get rid of genomic DNA contamination.

Complimentary DNA (cDNA) synthesis was done using SuperScript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative PCR (qPCR) was then carried out for xylA and xylB genes and housekeeping genes rpoD and 16S rRNA. Brilliant II SYBR Green (Stratagene) was used to track quantitative PCR carried out in Stratagene Mx3005P real time PCR system.

Results

Drastic Improvement in Xylose Fermentation by Adaptation

To develop a strain capable of fermenting xylose, an expression plasmid, pZMETX, was constructed to introduce four *E. coli* xylose-metabolism genes into *Z. mobilis* ZM4 (Fig. 1B). Despite successful detection of all four enzymatic activities, however, the transformant of ZM4 was unable to grow on xylose as sole carbon source. This was different from what was reported for similarly engineered strains (De Graaf et al., 1999; Zhang et al., 1995). ZM4/pZMETX was subsequently subjected to a brief adaptation. First, cells were grown in RM medium containing 4% glucose–1% xylose as carbon source and were subsequently subcultured in RM medium with mixed sugars with the ratio of glucose to xylose changed to 2.5–2.5%, 1–4%, and finally 5% xylose. The need for an initial adaptation was also reported by others (Viitanen et al., 2008; Zhang et al., 2002). The strain designated as A1 was the first strain that emerged through the adaptation process capable of growing on xylose as sole carbon source. However, xylose metabolism of this strain was sluggish and much slower than what was reported by a similarly engineered strain (Zhang et al., 1995) and much slower than its glucose metabolism.

To improve xylose fermentation, A1 was subjected to an adaptation process by serial dilution and subculturing in RM medium containing 5% xylose as sole carbon source. This process was carried out for 80 days involving 30 serial transfers. Initially, it took the cells approximately 96 h to reach late exponential phase (LEP) and an OD_{600} of approximately 0.6. As adaptation progressed, a gradual reduction in time taken to reach LEP and an increase in LEP OD_{600} were observed. By the 18th serial transfer, LEP OD_{600} reached about 1.2 (double the initial value), and no further increase in LEP OD_{600} in subsequent transfers was observed. The adaptive mutation process was terminated after 30th transfer and the adapted strain was designated as A3. The time taken for A3 to reach LEP was about 60 h, significantly lower than the initial 96 h, suggesting a significant acceleration in xylose metabolism.

Several experiments were run in bioreactors (see Materials and Methods Section) to characterize the adapted strain A3 and compared to its parental strain A1. In a mixed sugar fermentation containing 5% glucose and 5% xylose (5%G–

5%X), a typical diauxie behavior was observed in both strains (Fig. 2A). Glucose was consumed first, at an almost identical rate for the two strains. In contrast, the xylose fermentation between the two strains was drastically different. For A3, xylose was nearly consumed at about 32 h (residual xylose concentration at 0.11%), whereas for A1, the consumption of xylose was not complete even after more than 110 h (residual xylose concentration at 0.31%). As shown in Table I, maximum specific rate of xylose consumption by A3 was 1.8 g xylose/g DCW/h, three times higher than that for A1 (0.45 g/g/h).

Both strains exhibited identical growth curve on glucose, indicating glucose metabolism was not altered by adaptation. A3 was able to maintain the same growth rate upon transition from glucose to xylose, whereas A1 grew on xylose with much diminished rate. The highest dry cell mass reached was 2.8 and 2.0 mg/mL for A3 and A1, respectively (Fig. 2A). The difference suggests significant metabolic changes during xylose fermentation. Ethanol yield based on total sugars consumed was slightly higher for A3 (96.6%) than for A1 (93.7%) (Table I). Other fermentation byproducts were produced but at significantly lower level than ethanol. At the end of fermentation, the total amount of byproducts (lactic acid plus glycerol) produced were 0.2% and 0.15% for A3 and A1, respectively. The amount of xylitol produced by A3 was 0.06%, less than half of the amount produced by A1 (0.13%).

To further illustrate the superior performance by A3, following a mixed sugar fermentation as described above, a second dose of 5%G–5%X was fed to the cells when sugars were nearly exhausted. As shown in Figure 2B, A3 readily converted the second dose of sugars to ethanol, producing 9% ethanol with a combined yield of 93.4% from a total of 20% sugars. To our knowledge, this is the highest reported amount of ethanol produced by *Z. mobilis* in a mixed sugar fermentation. During the fermentation of the second dose, however, no significant cell growth was observed yielding only an additional cell mass increase of 0.445 g DCW/L. Additionally, xylose fermentation was significantly slower than that of the first period while glucose consumption rates remained high. Much more xylitol was produced during the second period, resulting in a final xylitol concentration of 0.26%. These data suggest that the ability of xylose fermentation was deteriorated in the second period, possibly due to accumulation of inhibitive metabolites (xylitol and ethanol) and depletion of nutrients as only sugars were fed in the second dose.

The superiority of A3 in xylose fermentation was further manifested in its ability to grow in 10% xylose as sole carbon source. As shown in Table I and Figure 2C, after 46 h of fermentation, nearly all of 10% xylose was consumed with only 0.33% xylose remaining, and 4.31% ethanol was produced with a yield of 88.2%. The maximum specific rate of xylose utilization was 3.44 g/g/h. In contrast, A1 grew very slowly on 10% xylose and after 46 h of fermentation, only 1% xylose was consumed. Even after 10 days, residual 3% xylose was measured. The final OD reached by A1 was five times lower than that of A3. These data are indicative of significantly improved ability in fermenting xylose at high concentration in A3. It is important to note that compared to mixed sugar fermentation, using xylose as sole carbon source poses significantly higher stress to cells. The higher the xylose concentration is, the more difficult it is for the cells to complete fermentation. This is due to primarily the accumulation of xylitol (see below sections). Thus, xylose fermentation at high concentration is a better measure for a strain's ability to use xylose than mixed sugar fermentation. Previously, the highest xylose concentration that supports cell growth was ~6% (Kim et al., 2000; Lawford and Rousseau, 1999), and in some cases, the fermentation was incomplete with significant amount of residual xylose (>1%; Lawford and Rousseau, 1999).

Taken together, the fermentation data clearly demonstrate that A3 outperforms A1 in xylose fermentation and its performance compares favorably with strains reported in the

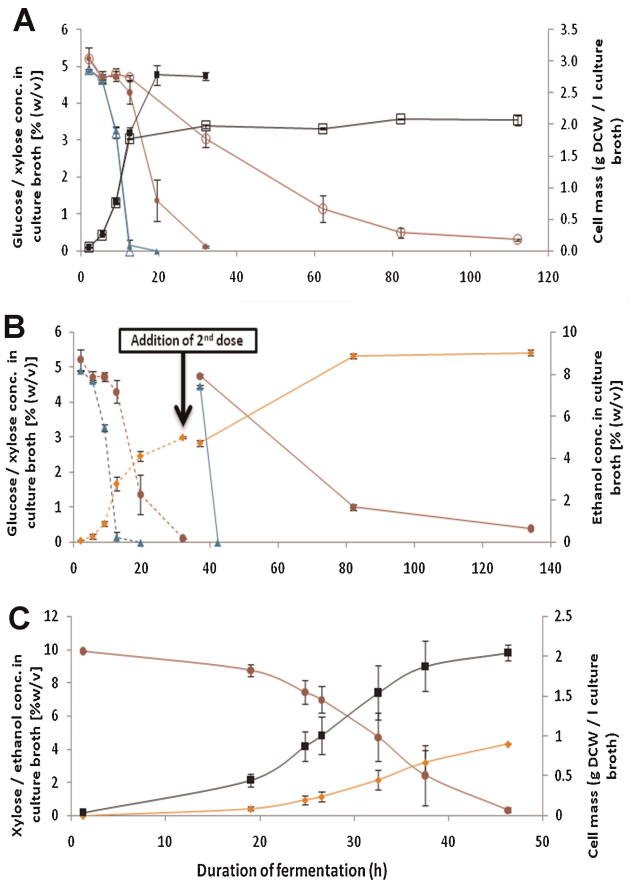

Figure 2. Fermentation profiles of A1 and A3. Three replicates were done for each condition. **A**: Mixed sugar fermentation of 5% glucose–5% xylose mixture. **B**: Fermentation of two doses of mixed sugars (5%G–5%X) by A3. Fermentation of the 1st dose is shown as dashed lines; the arrow indicates the point of addition of the 2nd dose of 5%G–5%X (only sugars). **C**: Fermentation of 10% xylose by A3. Black square (■)—cell mass, blue triangle (▲)—glucose, red circle (●)—xylose, orange diamond (◆)—ethanol. Filled symbols represent A3 while open symbols represent A1. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

Table I. Summary of fermentation parameters for A1 and A3.

Strain	Initial sugar conc. (%, w/v)	Max sp. rate of xylose consumption (g/g/h)	Final ethanol titer (%, w/v)	Ethanol yield (%)[a]	Final xylitol concentration (%, w/v)
A1	Mixed sugar (5%G–5%X)	0.45 ± 0.05	4.73 ± 0.06	93.7 ± 1.8	0.13 ± 0.01
A3	Mixed sugar (5%G–5%X)	1.80 ± 0.05	4.99 ± 0.06	96.6 ± 4.8	0.06 ± 0.00
A3	Two doses of mixed sugar (5%G–5%X)	ND	9.02 ± 0.15	93.4 ± 2.8	0.27 ± 0.01
A3	10% xylose	3.44 ± 0.25	4.31 ± 0.04	88.2 ± 0.1	0.11 ± 0.00

Three replicates were done for each experiment.
ND, not determined.
[a]Calculated based on consumed sugar (as a percentage of theoretical yield).

literature, illustrating the power of extended adaptation in strain improvement.

Both A1 and A3 were genetically stable as they exhibited the same growth and fermentation characteristics when cultured and tested under the same conditions over a 2-year period since their isolation.

Reduced Xylitol Production and Increased Xylitol Tolerance in A3

The greatly improved xylose fermentation by A3 prompts us to investigate the mechanisms responsible for the metabolic changes. At a metabolite level, it was observed that A3 produced much less xylitol during xylose fermentation. As previously suggested by other researchers (Feldmann et al., 1992; Jeon et al., 2005; Kim et al., 2000; Scangos and Reiner, 1979), xylitol formation and its toxicity may be an important factor. This was investigated as a potential reason for the improvement in A3. Figure 3 shows xylitol formation as a function of residual xylose during a mixed sugar fermentation (5%G–5%X). At the beginning of vigorous xylose fermentation (coinciding the exhaustion of glucose), xylitol formations were similar for both strains, at ~0.02%. However, during xylose fermentation the difference in xylitol formation between the two strains was significant (Fig. 3). This is in spite of a much higher xylose consumption rate for A3 as shown in Figure 2A. There was an apparent acceleration of xylitol production in A1 toward the end of the xylose fermentation, which contributed to the widening of the difference in xylitol formation between the two strains. As a result, at the end of the fermentation, twice as much xylitol was produced by A1 as by A3 (0.13% vs. 0.06%). These data suggest that adaptation altered the metabolism to disfavor the formation of xylitol in A3.

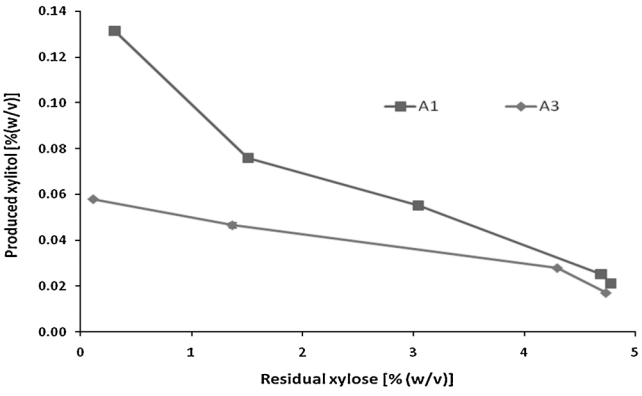

Figure 3. Xylitol produced by adapted strains as a function of residual xylose during mixed sugar (5%G–5%X) fermentation. Three replicates were done for each condition.

Additionally, it was observed that adaptation enhanced xylitol tolerance. In 5% xylose fermentation with exogenous xylitol at a concentration of 0.1%, growth rate was less affected in A3, with a reduction of about 20% (Table II), compared to a 50% decrease in specific growth rate for A1 under the same condition. The presence of xylitol at this concentration slows down xylose fermentation for both strains, but this is more so for A1 than A3, as indicated by the residual xylose at 42 h of fermentation (Table II). Sequence analysis of both xylose isomerase and xylulokinase genes showed no mutation in their respective genes, ruling out functional mutation in these two enzymes in conferring tolerance.

Taken together, these data suggested that adaptation caused significant metabolic changes that lead to less xylitol formation and better xylitol tolerance in adapted strain A3, two factors crucial for efficient xylose utilization.

Table II. Effects of exogenous xylitol (0.1%) on xylose fermentation.

	Avg[a] specific growth rate (h^{-1})	Reduction in sp. growth rate for cells cultured in 0.1% xylitol	Residual xylose[b] (%, w/v)
A1 (0% xylitol)	0.069 ± 0.002	47.8%	1.5 ± 0.2
A1 (0.1% xylitol)	0.036 ± 0.001		3.8 ± 0.1
A3 (0% xylitol)	0.094 ± 0.001	20.2%	0
A3 (0.1% xylitol)	0.075 ± 0.005		1.3 ± 0.1

Cells were grown on 5% (w/v) xylose in 20 mL capped vials. Three replicates were done for each condition.
[a]Averaged value over the first 42 h of cell growth. Initial OD at ~0.05.
[b]Measured at 42 h of fermentation.

Higher Xylose Isomerase Activity in A3

Activity assays were carried out for four xylose metabolizing enzymes (xylose isomerase (XI), xylulokinase (XK), transaldolase (Tal), and transketolase (Tkt)) and two enzymes linking the engineered pentose phosphate pathway to glycolysis, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and phosphoglucose isomerase (PGI). Table III shows the enzymatic activities measured in cell-free extracts of A1 and A3 under mixed sugar fermentation (5%G–5%X) at different stages of fermentation. Xylose isomerase activity in both strains lags far behind other five enzymes, from two- to threefold up to three orders of magnitude. The significantly low XI activity relative to other heterologous xylose-metabolizing enzymes was previously observed by others (Zhang et al., 2002). It can be argued that based upon the results, XI may represent a metabolic bottleneck.

A comparison of XI activity between the two strains reveals that XI activity in A3 is significantly higher (4–8 times) than A1 when fermenting 5%G–5%X mixture (Table III). Additionally, it appears the difference widens as fermentation progresses (Table III). For other enzymes measured, the differences between the two strains were not as dramatic as XI (Table III). The significance of smaller differences in these enzymes may not be as important as XI, as XI activity was the lowest. Apparently, adaptation resulted in an acceleration of the slowest step in xylose fermentation. It is important to note, however, even after the increased XI activity in A3, XI activity remains to be low, as compared to other measured enzyme activities.

Although xylose isomerase inhibition by xylitol was not experimentally shown for XI from *E. coli*, it is thought to be the case based on an in vitro study on *Arthrobacter* XI (Smith et al., 1991). As the significant difference in activities between the two strains was observed even when xylitol concentrations were similar (Ph1 and Ph2, Table III, Fig. 3), it is unlikely that the difference in XI activity between the two strains is due to inhibition of XI by xylitol in this case. To identify possible mutation responsible for the increased activity of XI, the recombinant plasmid pZMETX (Fig. 1B) in both strains was sequenced. Compared to the plasmid isolated from A1, the only mutation found from the plasmid isolated from A3 was within the Ppdc promoter region. The single base pair mutation, G to A, occurred at −23 bp (23 base pair upstream from the start codon of xylA). As the mutation occurred between the major transcription initiation site (−46 bp) and potential ribosome binding site (rbs) (−10 to −7) (Conway et al., 1987), it is likely to affect the transcriptions of xylA–xylB. However, RT-PCR experiment failed to detect any significant changes for either xylA or xylB between A1 and A3 (data not shown). It is possible, however, the mutation may affect some post-transcription events, leading to increased expression of XI. Unfortunately, changes in expression in XI could not be confirmed by SDS–PAGE since low levels of XI in both A1 and A3 prevented detection by SDS–PAGE. Future studies are needed to clarify the role of the single mutation on its expression and activity.

Discussion

Although the feasibility of lignocellulosic ethanol has long been demonstrated, developing cost-effective technology to realize the potential on a large scale has been a challenge. In this work, we sought to improve ethanol production by enhancing the ability of biocatalyst to use xylose. Specifically, the ability to ferment xylose at high concentration and reducing fermentation time are important engineering targets as they impact productivity and downstream processing. As demonstrated here, through an adaptation process, a strain (A3) with significantly improved traits was obtained. First the xylose consumption rate in A3 was significantly increased compared to its parental strain, A1, resulting in significantly reduced fermentation time in mixed sugar (5%G–5%X) fermentation, from over 110 h to about 35 h. Additionally, the improved xylose fermentation was manifested by its ability to ferment high concentration of xylose. In fact, A3 was able to grow with 10% xylose as sole carbon source, which was the highest concentration ever reported. The improved xylose fermentation and the ability to use high concentration of xylose can be used to achieve high product concentration. As demonstrated here, fermenting two doses of 5%G–5%X by A3 allowed ethanol to be accumulated to

Table III. Enzymatic activities[a] (in mU/mg cell protein) in cell-free extracts of A1 and A3 harvested from mixed sugar fermentation (5%G–5%X).

Strain name (time of harvest)	XI	XK	Tal	Tkt	GAPDH	PGI
A1 (Ph 1)	23 ± 0	1,700 ± 200	610 ± 30	350 ± 70	1,200 ± 100	1,900 ± 200
A3 (Ph 1)	105 ± 7	2,300 ± 100	540 ± 40	430 ± 70	1,500 ± 200	2,400 ± 100
A1 (Ph 2)	31 ± 1	1,800 ± 200	520 ± 70	280 ± 10	1,500 ± 200	3,300 ± 200
A3 (Ph 2)	162 ± 28	2,200 ± 400	700 ± 40	380 ± 70	1,800 ± 400	3,500 ± 200
A1 (Ph 3)	26 ± 4	2,300 ± 400	670 ± 70	450 ± 60	2,000 ± 100	2,900 ± 100
A3 (Ph 3)	210 ± 26	2,300 ± 500	810 ± 20	440 ± 10	2,500 ± 100	4,400 ± 200

Cells were harvested during exponential growth on glucose (Ph 1), at the onset of xylose fermentation (Ph 2) and near the exhaustion of all xylose (Ph 3). XI, xylose isomerase; XK, xylulokinase; Tal, transaldolase; Tkt, transketolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGI, phosphoglucose isomerase.

[a]Three replicates were done for each condition. XI assay data for A1 and A3 significantly different ($P < 0.05$). For remaining enzymes, assay data not significantly different for A1 and A3 except for Ph 3 data for GAPDH and PGI.

9%, the highest ever shown for this organism in mixed sugar fermentation. The combined benefits of reduced fermentation time and increased ethanol concentration should generate a cost advantage for bioethanol processes employing A3.

Altered xylitol metabolism was responsible, at least partially, for the improvement. At least two pathways were implicated for xylitol formation in *Z. mobilis*. The first is analogous to eukaryotic aldose reductase (Feldmann et al., 1992). The second is catalyzed by the glucose–fructose oxidoreductase (GFOR) (Viitanen et al., 2008; Zhang et al., 2009). Both enzymes are NADPH dependent but the cofactor in GFOR is tightly bound to the enzyme. An analysis of the whole cell extract for the aldose reductase activity showed that in both A1 and A3, the aldose reductase activity was barely detectable, whereas the wild-type strain ZM4 carrying an empty plasmid (without the xylose-metabolism heterologous genes) gave an activity of 22 mU/mg-proteins. The xylitol formation for the control was also three times higher at 0.06% than both A1 and A3. This suggests that adaptation cause a reduction of aldose reductase activity in both strains, which is at least partially responsible for the reduction of the xylitol formation in A1 and A3, although this does not explain the difference between A1 and A3. In light of DuPont patent (Viitanen et al., 2008), the residual xylitol formation may be due to GFOR. However, we found no mutation in GFOR gene and its promoter region in both strains. Therefore, while evidence pointed to reduced xylitol formation in A3 as key for improvement, further studies are needed to clarify the role of various mechanisms in xylitol formation. It is possible that other chromosomal mutations contribute to the different rates of xylitol formation between the two strains. It is also possible that A3 could benefit from other chromosomal mutations unrelated to xylitol synthesis.

The work presented here illustrates the power of adaptation in strain improvement. While selection pressure applied in the adaptation is high concentration of xylose (5%), not xylitol, xylitol tolerance and reduced xylitol formation were selected. In addition, the increased XI activity favors the reduction of xylitol effects, by channeling more xylose to ethanol instead of xylitol. This further confirms what has been suggested by other researchers that xylitol metabolism is the key to efficient use of xylose in this organism. This study is thus a good example to demonstrate that adaptation not only is useful in improving strains but also could be equally well used to pinpoint bottlenecks in metabolically engineered strains. Identified targets from adaptation could be further improved through metabolic engineering. In this case, xylitol formation could be further reduced by knockout genes for GFOR and aldose reductase once identified. Additionally, further increase of xylose isomerase expression will also be helpful in reducing xylitol formation. Furthermore, even in GFOR knockout strain, or reduced aldose reductase strains, xylitol formation was not eliminated. This may suggest a redox imbalance that could be addressed by metabolic engineering. Therefore, adaptation and metabolic engineering could be used synergistically in developing industrially useful strains.

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