1 Mutation in *galP* improved fermentation of mixed sugars to succinate using
2 engineered *Escherichia coli* AS1600a and AM1 mineral salts medium

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ABSTRACT

Escherichia coli KJ122 was engineered to produce succinate from glucose using the wild type GalP for glucose uptake instead of the native phosphotransferase system (*ptsI* mutation). This strain now ferments 10% xylose poorly. Mutants were selected by serial transfers in AM1 mineral salts medium with 10% xylose. Clones from this population all exhibited a similar improvement, co-fermentation of an equal mixture of xylose and glucose. One of these, AS1600a, produced 84.26 ± 1.37 g/L succinate, equivalent to that produced by the parent (KJ122) from 10% glucose (85.46 ± 1.78 g/L). AS1600a was sequenced and found to contain a mutation in galactose permease (GalP, G236D). This mutation was shown to be responsible for the improvement in fermentation using KJ Δ galP as the host and expression vectors with native *galP* and with mutant *galP**. Strain AS1600a and KJ Δ galP(pLOI5746; *galP**) also co-fermented a mixture of glucose, xylose, arabinose, and galactose in sugarcane bagasse hydrolysate using mineral salts medium.

Keywords: succinate, metabolic evolution, furfural, sugarcane bagasse, xylose, arabinose

1. Introduction

Sugars derived from lignocellulosic biomass represent an attractive feedstock for the fermentative production of chemical and plastics, replacing petroleum and eliminating competition for food (starch-based products). However, harsh pretreatments are required to soften this structure and increase access by cellulase enzymes (Keating et al., 2014). Dilute acid pretreatment can provide near quantitative hydrolysis of hemicellulose polymers into monomeric sugars, but also creates toxic side products from sugars and lignin. Side products such as furfural, a dehydration product of pentose sugars, strongly inhibit growth and fermentation (Miller et al., 2009).

Several approaches are available to reduce the toxicity of dilute acid hydrolysates for ethanol production. Including evaporation of volatiles under vacuum (Frazer and McCaskey, 1989; Chandel et al., 2013; Geddes et al. 2015), addition of sodium metabisulfite (Nieves et al., 2011), and base-treatments with ammonia or lime (Martinez et al., 2000, Geddes et al., 2013). Recently, Geddes et al. (2015) evaluated that effectiveness of combining treatments (vacuum evaporation, laccase, high pH, bisulfite, and micro-aeration) to eliminate all inhibitory activity in dilute hemicelluloses hydrolysate. Removing all toxins in this way, however, may increase process cost and reduce yields of fermentable sugars (Keating et al., 2014).

Genetic improvement of biocatalysts arguably offers the most cost-effective approach to mitigate inhibitors in lignocellulose hydrolysates and to improve performance during fermentation. Many useful genes have been described for furfural tolerance in *E. coli* (Wang et al., 2013; Miller et al., 2009) during ethanol production. Optimal biocatalysts must be able to co-ferment sugar mixtures (primarily glucose and xylose) within a single vessel, eliminating the need for liquid/solid separation or

expensive treatments to mitigate toxins. No such strains are currently available for the production of succinate, a commercial intermediate for plastics, surfactants, green solvents, and detergents (Zeikus et al., 1999).

E. coli KJ122 was originally developed to ferment pure glucose streams from starch into succinate using GalP for glucose uptake (Jantama et al., 2008b; Zhang et al., 2009). However, previous studies have reported that this strain performs poorly with xylose (Wang et al., 2013). Xylose is the most abundant sugar in dilute acid hydrolysates of sugarcane bagasse. Effective use of xylose, glucose, and other sugars by biocatalysts is desirable for commercialization of lignocellulosic feedstocks. In this paper, we describe a derivative of KJ122 (strain AS1600a) that ferments xylose to completion as the sole carbon source, in sugar mixtures, and in a dilute acid hydrolysate of sugarcane bagasse using a simple mineral salts medium.

2. Materials and Methods

2.1 Strains and plasmids

Strains, plasmids, and primers used in this study are listed in Table 1. All chromosomal modifications were made in *E. coli* KJ122. Luria Bertani medium was used for plasmid constructions (Top10F' host), *galP* gene deletion (KJ122) and plasmid transformation of succinate-producing strains. After genetic manipulations, succinate biocatalysts were grown in AM1 mineral salts medium (Jantama et al., 2008b; Martinez et al., 2007).

2.2 Isolation, sequencing, and comparison of chromosomal DNA.

Genomic DNA samples from *E. coli* (ATCC 8739), KJ122, and AS1600a were purified according to the bacterial genomic DNA protocol from the DOE Joint Genome Institute (<http://jgi.doe.gov>). Next-generation sequencing was performed using Illumina paired-end technology (150 bp read length). Sequencing and bioinformatics was provided by the Tufts University Core Facility (Boston, MA). Sequences were aligned and compared (CLC Sequence Viewer, Qiagen, Valencia, CA) using *E. coli* ATCC 8739 (Accession number NC_010468.1, NCBI) as the template.

2.3 Construction of expression vector pLOI5883.

Many plasmids that are stable in LB medium are less stable in AM1 mineral salts medium. A stable expression vector (pLOI5883) was constructed by replacing the pBR322 *oriR* replicon in pTrc99a with the RSF1010 replicon from pLOI707EH (Arfmann et al., 1992). Primer pairs RSF1010*rep* and pTrc99aΔ*oriR* were used to amplify the RSF1010 replicon and the pTrc99a backbone, omitting *oriR*. After amplification, both fragments were digested with SpeI and ligated (Figure S2). The resulting plasmid, pLOI5883, was confirmed by Sanger sequencing (Interdisciplinary Center for Biotechnology Research, University of Florida). After 5 serial transfers of KJ122 (pLOI5883) in AM1 glucose (50 g/L) broth without antibiotics, 100% of colonies retained the plasmid.

2.4 Cloning *galP* and *galP** into expression vectors.

The *galP* gene was amplified from *E. coli* KJ122 (*galP*) and AS1600a (*galP**) using primer pair galP3 (Table 1) and Phusion polymerase (New England Biolabs, Ipswich,

MA). Amplified fragments were cloned into pLOI5883 between the NdeI and PstI sites. Resulting plasmids were designated pLOI5746 and pLOI5747 (Figure S2), expressing the mutated *galP** and native *galP* genes, respectively. Constructions were confirmed by Sanger sequencing. Expression was induced by adding 10 μ M IPTG.

2.5 Construction of vector (pLOI5899) for chromosomal integration.

Previous studies have used pLOI4162 (Jantama et al., 2008b) containing a *cat-sacB* cassette for selection of integration (chloramphenicol resistance) and counter selection (resistance to sucrose). Expression of *sacB* (dextran-sucrase) is lethal for *E. coli* in the presence of sucrose. The native *sacB* terminator was added to this cassette to reduce transcription of downstream genes. The backbone of pLOI4162 was amplified using primer pair 4162 (omitting the *sacB* gene). The native *sacB* gene including transcriptional terminator was amplified from *Bacillus subtilis* YB886 using primer pair 5899. After digestion with XbaI and SacI, the two amplified fragments were ligated to make pLOI5899 (Figure S3). Construction was confirmed by Sanger sequencing.

2.6 Deletion of *galP* in KJ122

Methods for chromosomal deletions have been previously described using Red recombinase technology (Datsenko and Wanner, 2000; Jantama et al., 2008b). Briefly, the *cat-sacB* cassette in pLOI5899 was amplified using primers that bridged the *cat-sacB* cassette and chromosomal region 5' and 3' to *galP* (primer pair galPdel). The resulting amplified fragment was integrated into KJ122 by double homologous recombination with selection for chloramphenicol resistance (KJ122 Δ *galP::cat-sacB*),

designated strain KJΔ*galP*. Correct integration was confirmed by amplification and sequencing.

2.7 Fermentation

Fermentations were conducted in pH-controlled vessels (500 mL) with a 300 mL working volume (37 °C, 150 rpm). Medium was maintained (pH 7.0) by automatic addition of a mixture of 6 N KOH and 3 M K₂CO₃ (1:4 ratio) (Jantama et al., 2008b). A low salt medium, AM1 (4.2 g/L total salt, Martinez et al., 2007) supplemented with 1 mM betaine, 100 mM KHCO₃ (Jantama et al., 2008b), and sugar (as indicated) was used for fermentation. Seed cultures were grown overnight (16 h) in AM1 medium supplemented with 5% sugar. Fermentations were run with glucose, xylose, or a mixture of the two at either 5% total sugar or 10% total sugar. This total sugar concentration (100 g sugar/L) was can be readily achieved from a 15% w/v slurry of sugarcane bagasse by a combination of dilute acid pretreatment and cellulase. Fermentations were inoculated to an initial OD_{550nm} of 0.1 (33 mg DCW/L). These were sampled and monitored for up to 120 h in some cases.

2.8 Metabolic evolution to select for improved xylose utilization

Strain KJ122 was repeatedly sub-cultured in AM1 medium containing 10% xylose until performance after 24 h was equivalent to KJ122 with 10% glucose (Supplemental Figure S1; Figure 1A and Figure 1B) using pH-controlled fermenters (500 mL) with a 300 mL working volume (pH 7.0, 37 °C, and 150 rpm) (Jantama et al., 2008a). After 16 transfers, broth was spread on solid medium. Clones were tested individually for xylose utilization. One was selected for further work (designated AS1600a) and stored at -80°C as glycerol stocks (40% glycerol).

2.9 Preparation of sugarcane bagasse hydrolysate

Dilute acid hydrolysates of sugarcane bagasse were prepared at the University of Florida Biofuels Pilot Plant as described previously (8 kg phosphoric acid per tonne bagasse, 5 min, 190 °C; Nieves et al., 2011). Hemicellulose syrup (hydrolysate) was recovered using a screw press, discarding solids. After removal of fine particulates with a Whatman GF/D glass fiber filter, clarified hydrolysate was stored at 4 °C (pH 3.0). The average composition of sugarcane bagasse hydrolysates was 44 g/L xylose, 5 g/L glucose, 8 g/L arabinose, 4 g/L galactose, 5 g/L acetate, 2 g/L furfural, and trace amounts of hydroxymethyl furfural.

2.10 Detoxification of sugarcane bagasse hydrolysate

Unless indicated otherwise, half of the weight of clarified bagasse hydrolysate was evaporated under vacuum at 55 °C to remove volatile compounds by using a rotary evaporator (Buchi Rotavapor R110 evaporator, Flawil Switzerland) equipped with a Cole Palmer aspirator pump Model 7049-00 (Chicago, Illinois) (Geddes et al., 2015). The resulting concentrate was restored to original weight by adding sterile deionized water. Vacuum-treated hydrolysate was adjusted to pH 9.0 by addition of ammonium hydroxide (5N NH₄OH) and allowed to remain at room temperature for 16 h before inoculation. During this period, the broth pH declines to near pH 7. Stock solutions of bisulfite were freshly prepared and added immediately prior to inoculation (2 mM sodium metabisulfite, final concentration) (Nieves et al., 2011).

2.11 Analyses

(b)

Cell mass, organic acids, furfural, and sugars were measured during fermentation. Cell mass was estimated from OD_{550nm} (0.33 mg of cell dry weight/mL/OD) using a Bausch & Lomb Spectronic 70 spectrophotometer (Jantama et al., 2008b). OD was not measured in hydrolysate fermentations due to color (Geddes et al., 2014, 2015). Sugars, furans, and organic acids were analyzed by two high-performance liquid chromatography (HPLC) systems (Agilent Technologies 1200) as described previously (Geddes et al., 2014). Sugars and furan were analyzed using a BioRad (Hercules, CA) Aminex HPX-87P ion exclusion column (80 °C; nano-pure water as the mobile phase, 0.6 mL/min). Organic acids were analyzed using a BioRad Aminex HPX-87H column (45 °C; 4 mM H₂SO₄ as the mobile phase, 0.4 mL/min).

2.12 Statistical methods

Analysis of variance (ANOVA) was conducted using SPSS software (SPSS 17.0 for Windows; SPSS, Inc., Chicago, IL, USA). Results from at least 3 tests are reported as averages with standard deviations. Differences among mean values were established using Duncan's multiple range tests at 95% significance level.

3. Results and Discussion

3.1 Fermentation of glucose and xylose by strain KJ122

Strain KJ122 was developed from *E. coli* ATCC8739 for the fermentation of pure glucose into high titers of succinate (Jantama et al., 2008b) but has been reported to ferment xylose slowly (Wang et al., 2013). This problem was confirmed using 10% sugars and served as a starting point for strain improvement (Figure 2A and 2B). Strain KJ122 exhibited a lag of 48 h with 10% xylose and left half of the xylose unfermented

after 120 h (Figure 1A and 1B; Table 2). In contrast, strain KJ122 fermented 10% glucose to completion without a lag (Figure 1A, 1B, and 2B). Reducing the xylose concentration from 10% to 5% improved fermentation by reducing the lag to 24 h (Figure 2C) and fermenting 5% xylose to substantial completion within 96 h. After fermentation for 120 h, 44.17 ± 0.78 g/L and 85.46 ± 1.78 g/L succinate were produced from 5% and 10% glucose, respectively. Succinate production from 10% glucose (85.46 ± 1.78 g/L) was 2.5 times higher than succinate production from 10% xylose (37.49 ± 1.72 g/L). Succinate yields with KJ122 and glucose were also higher than with xylose, 0.88 g/g glucose (metabolized) as compared to 0.81 g/g xylose (Table 2). More effective fermentation of both xylose and glucose is needed to allow the use of mixed sugars from lignocellulose as a feedstock.

3.2 Metabolic evolution for improved xylose utilization

Metabolic evolution has been previously used to improve biocatalysts for many fermentation products including ethanol (Olsson et al., 2007; Yomano et al., 2008), D-lactate (Utrilla et al., 2012; Zhou et al., 2003), and succinate (Jantama et al., 2008a). This method was applied to *E. coli* KJ122 to develop an improved biocatalyst for the fermentation of xylose to succinate (Figure 1A and 1B; Supplemental Figure S1).

Poor growth of KJ122 in AM1 medium containing 10% xylose was used as the basis for selection. The initial inoculum grew very little for 3 days. After 3 days, this culture was transferred to fresh medium, and transferred at 24-h intervals thereafter. Succinate production and growth (16 serial transfers in 10% xylose) exceeded that of KJ122 with 10% glucose (Figures 1A, 1B, and 2B; Table 2; Supplemental Figure S1). Colonies were isolated from solid medium and tested for succinate production in pH-controlled

1 fermentations with 10% xylose. All appeared similar and one was selected for further
2 study, designated AS1600a (Figure 3A). This strain produced 84.26 ± 1.37 g/L succinate
3 from 10% xylose with a yield of 0.88 g/g sugar metabolized) (Table 2). Succinate yield
4 with AS1600a and xylose was equivalent to the parent with 10% glucose (85.46 ± 1.78
5 g/L). AS1600a exhibited a 3-fold improvement in volumetric productivity with 10%
6 xylose (0.96 g/L/h) as compared to KJ122 (0.31 g/L/h). Unlike KJ122, strain AS1600a
7 grew on 10% xylose without a lag, closely resembling the fermentation pattern with
8 KJ122 and 10% glucose. With strain AS1600a, xylose was fermented more effectively
9 than glucose.

10 Differences were also observed during the fermentation of an equal mixture of
11 glucose and xylose (10% total sugar). The mutant AS1600a co-fermented both glucose
12 and xylose to near completion without lags and at similar rates (Figure 3D). In contrast,
13 the parent strain KJ122 fermented glucose to completion but fermented only half of the
14 xylose (Figure 3D; Table 2). With these two sugars, the combined rates of xylose and
15 glucose fermentation to succinate (maximum productivities) for AS1600a and KJ122
16 were similar to 10% xylose alone and 10% glucose alone, respectively.

18 3.3 A single mutation in *galP* is sufficient to improve xylose metabolism in KJ122.

19 Chromosomal DNA from the parent KJ122 and the mutant AS1600a was isolated,
20 sequenced, and compared. Only 2 new mutations were found, G236D in *galP* (glycine
21 to aspartate) and L287Q in a cryptic 4.5 kbp *rhs*-like gene (leucine to glutamine), absent
22 from many *E. coli* strains. Each mutation resulted from a single nucleotide change
23 within the coding region. The native *galP* permease is important for glucose uptake by
24 KJ122 (Zhang et al., 2009), due to a mutation in *ptsI* that blocks glucose uptake by the

1 native phosphotransferase system. This *ptsI* mutation also conserved
2 phosphoenolpyruvate for succinate production and increased ATP yield when coupled
3 with pyruvate carboxykinase (up-regulated in KJ122).

4 The *galP** mutation in AS1600a was investigated as a possible cause for improved
5 xylose metabolism. The coding region for *galP* was deleted from KJ122 (parent) by
6 double homologous recombination to make KJΔ*galP*. This strain grew poorly in AM1
7 medium with 10% glucose or 10% xylose and was maintained on LB glucose plates.
8 Growth on AM1 glucose or xylose was restored by supplying GalP activity from
9 plasmids. Strains KJΔ*galP*(pLOI5747; wild type *galP*) and KJΔ*galP*(pLOI5746; mutant
10 *galP**) were compared to the AS1600a (mutant) during fermentation with 10% xylose
11 alone, 10% glucose alone, and with a mixture of 5% glucose and 5% xylose (Figure 2,
12 Table 2). Strains KJΔ*galP*(pLOI5746; *galP**) and AS1600a were very similar. Both
13 fermented 10% xylose to succinate (without lags) as effectively as the parent KJ122 and
14 KJΔ*galP*(pLOI5747; wild type *galP*) fermented 10% glucose (Table 2). However,
15 strains with the *galP** were less effective than the parent with 10% glucose alone. The
16 *galP*-deleted strain with the *galP** mutant plasmid (pLOI5746) and AS1600a co-
17 fermented the mixture of glucose and xylose (5% each) while the parent KJ122 and
18 KJΔ*galP* strain with pLOI5747 (wild type *galP*) used glucose preferentially. With these
19 two strains, almost half of the xylose remained after 120 h. Expressing the *galP**
20 mutation (G236D) in KJΔ*galP* fully duplicated the xylose utilization phenotype of
21 mutant AS1600a, without the mutation in the cryptic *rhs*-like mutant gene.

22 Sugar transporters often utilize multiple sugars with differing affinities. GalP
23 appears to be a particularly very versatile proton symport, also transporting fructose,
24 glucose, and lactose (Zhang et al., 2009; Zheng et al., 2010). Expression of an

1 unmutated *galP* was able to replace the native phosphotransferase system for glucose in
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1 unmutated *galP* was able to replace the native phosphotransferase system for glucose in
2 *E. coli* KJ122 (Jantama et al., 2008b; Zhang et al., 2009; Hernández-Montalvo et al.,
3 2003), restoring growth and succinate production. Overexpression of native *galP* from a
4 high copy vector increased both glucose and xylose metabolism in *Enterobacter*
5 *cloacea* engineered for butanediol production (Li et al., 2015).

3.4 Co-fermentation of sugars in lignocellulose hydrolysate from sugarcane bagasse

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8 Mutant strain AS1600a was compared to KJ122 (parent) using sugarcane bagasse as
9 a substrate. Filtered sugarcane bagasse hydrolysate (55 g/L total sugar) was used to
10 simulate hydrolyzed lignocellulose by adding 150 g/L glucose as a replacement for
11 hydrolyzed cellulose fiber and diluting with an equal volume of water. The resulting
12 broth contained mostly glucose and xylose with smaller amounts of arabinose and
13 galactose, together with inhibitors from side reactions during dilute acid pretreatment.
14 Concentrations of sugars (100 g/L) and inhibitors are equivalent to hydrolysate prepared
15 from a slurry of 15% sugarcane bagasse (dry weight). A combination of treatments was
16 used to mitigate toxicity in this broth based on prior studies with ethanologenic *E. coli*
17 (Geddes et al., 2015). Furfural and other volatiles were removed by vacuum
18 evaporation. At large scale, both may be useful as co-products. Other inhibitors were
19 mitigated by pH 9 treatment with ammonia (16 h incubation) and addition of 2 mM
20 sodium metabisulfite. Phosphate used for dilute acid pretreatment and ammonia added
21 for neutralization served as macronutrients. Trace metals and magnesium sulfate were
22 added to complete the AM1 medium.

23 All sugars were co-metabolized to differing extents. The parent KJ122 fermented
24 the small amounts of galactose and arabinose to completion in sugarcane hydrolysate

within 48 and 96 h, respectively. The most abundant sugar glucose was fully
 metabolized after 144 h, but 80% of the xylose remained unfermented after 144 h
 (Figure 4A and 4C). In contrast, strain AS1600a containing the *galP** mutation
 fermented galactose, arabinose and xylose to near completion (Figure 4B and 4D), but
 left 20% of the glucose unused. With AS1600a, fermentations times were reduced by 24
 h each for galactose and arabinose, much more so for xylose. Interestingly, the weights
 of unfermented sugars in hydrolysate (glucose with AS1600a and xylose with KJ122)
 were similar for both strains. The differences in arabinose and xylose utilization were
 surprising. Except for uptake, reversible xylose isomerization, and initial
 phosphorylation, remaining genes encoding metabolism are the same for both pentose
 sugars. Differences in rate of utilization must reside with these early steps. The change
 in sugar preference in AS1600a and KJΔ*galP*(pLOI5746) is presumed to cause a change
 in GalP structure, consistent with uptake as the determining event.

3.5 Mutation in cryptic gene related to *rhs* C-terminal tip in *E. coli* W (ATCC9637).

A second point mutation was found in AS1600a, within a large reading frame (4.5
 kbp) that is 90% identical to a cryptic gene in *E. coli* W (ATCC9637), a putative *rhs* C-
 terminal tip (Archer et al., 2011). This gene is absent in most *E. coli* strains, including
 K12 (MG1655). Little is known about the activity and function of this protein. Some
rhs-like proteins have been associated with intercellular competition and cell
 inactivation. Amplifying this gene by PCR and cloning have proven difficult due to the
 highly repeated regions of sequence and length. This mutation was not required for
 reconstruction of xylose utilization in a KJΔ*galP*. The *galP** mutation alone was

sufficient to fully confer the improvements in xylose fermentation without the *rhs*-like mutant. Study of the *rhs* mutation will be the subject of future investigations.

3.6 Comparison of xylose fermentation by *E. coli* strains engineered for succinate.

Many mutants of *E. coli* have been constructed that are able to effectively ferment xylose alone, mixtures of sugars, and lignocellulose hydrolysates (corn stalk, sugarcane bagasse, etc). However, all prior studies have used complex medium supplements such as tryptone, yeast extract, corn steep liquor, or Luria broth (Table 3). Product yields have been quite good on a sugar basis in some cases, with product titers of over 50 g/L. However, these complex nutrients may be prohibitively expensive for most commercial processes. Others use multistep processes with a separate aerobic growth phase followed by a production phase (Andersson et al., 2007; Wang et al., 2011; Liu et al., 2013a) or repetitive fermentations (Liang et al., 2013; cell recycle) which may reduce media costs. Our work using AM1 mineral salts medium (Martinez et al., 2007) in simple batch fermentations achieved titers of over 70 g/L with pure xylose, an equal weight mixture of glucose and xylose, and from sugarcane bagasse hydrolysate with yield above 0.8 g/g sugar. The use of mineral salts medium offers two main advantages over complex supplements: reduction in media cost and simpler purification.

4. Conclusion

E. coli strain KJ122 was designed for the fermentation of glucose to succinate (Jantama et al., 2008b; Wang et al., 2013) but performed poorly with 10% xylose in AM1 mineral salts medium. An improved strain was easily obtained by growth-based selection, designated AS1600a. This mutant also co-fermented a glucose-xylose

1 mixture, and a mixture of 4 sugars in sugarcane bagasse hydrolysate. A single mutation
2 in *galP* (G236D) was shown to be responsible for the improvement in pentose
3 fermentation. This *galP** mutant gene may be useful for the improvement of sugar
4 metabolism in other biocatalysts.

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Figure Legends:

Figure 1. Metabolic evolution of *E. coli* KJ122 in xylose for succinate production.

Red line and squares indicate the source for isolation of AS1600a. A. Succinate; B. Cell mass.

Figure 2. Succinate production from glucose and xylose by *E. coli* KJ122 (parent) in AM1 mineral salts medium. A. Fermentation of 10% xylose; B. Fermentation of 10%

glucose; C. Fermentation of a xylose (5%); D. Fermentation of a sugar mixture containing 5% xylose and 5% glucose. Symbols for all: total sugar (open square), xylose (open circle), glucose (open triangle), biomass (filled circle), succinate (filled triangle), acetate (filled square).

Figure 3. Effect of *galP** mutation on succinate production from glucose and xylose.

A. AS1600a (improved mutant) with 10% xylose; B. KJΔ*galP*(pLOI5747 harboring wild type *galP*) with 10% xylose; C. KJΔ*galP*(pLOI5746 harboring *galP** mutation);

D. AS1600a (improved mutant) with sugar mixture (5% xylose and 5% glucose); E.

KJΔ*galP*(pLOI5747 harboring wild type *galP*) with sugar mixture (5% xylose and 5%

glucose); F. KJΔ*galP*(pLOI5746 harboring *galP** mutation). Symbols for all: mixed

sugars (open square), xylose (open circle), glucose (open triangle), biomass (filled

circle), succinate (filled triangle), acetate (filled square).

Figure 4. Fermentation of mixed sugars in sugarcane bagasse hydrolysate by KJ122

(parent) and AS1600a (improved mutant). A. Succinate production by KJ122. B.

Succinate production by AS1600a. C. Sugar utilization by KJ122. D. Sugar utilization

by AS1600a. Symbols for all: total sugars (open square), xylose (open circle), glucose

(open triangle), arabinose (open inverted triangle), galactose (open diamond), succinate

(filled triangle), acetate (filled square).

Table 1. Strains, plasmids and primers used in this study

Strains and plasmids	Relevant characteristics or sequence	Source or references
Strains		
<i>E. coli</i> KJ122	<i>E. coli</i> ATCC 8739 (Δ ldhA, Δ adhE, Δ ackA, Δ (focA-pflB) Δ mgsA, Δ poxB, Δ tdcDE, Δ citF, Δ aspC, Δ sfcA, pck*, ptsI*)	Jantama et al., 2008b; Zhang et al., 2009
<i>E. coli</i> KJ Δ galP	KJ122 Δ galP::cat-sacB	This study
<i>E. coli</i> AS1600a	Clone from 16 th transfer of <i>E. coli</i> KJ122 in 10% xylose containing point mutations in galP (galP*) and an rhs-like gene	This study
<i>E. coli</i> Top10F'	Host used for plasmid construction	Thermo Fisher
<i>Bacillus subtilis</i>	Strain YB886 Source of sacB gene with native terminator	Laboratory collection
Plasmids		
pTrc99a	pTrc bla oriR rrnB lacI ^q	Laboratory collection
pCR2.1-TOPO	ori bla lacI ^q , vector for constructions	Thermo Fisher
pLOI707EH	Source of RSF1010 replicon	Arfman et al., 1992
pLOI4162	Integration vector	Jantama et al., 2008b
pLOI5899	Improved integration vector with sacB terminator	This study
pLOI5883	Expression vector, RSF1010 rep pTrc bla rrnB lacI ^q	This study
pLOI5746	pLOI5883, expression of galP* (mutant)	This study
pLOI5747	pLOI5883, expression of galP (native gene)	This study
Primers		
RSF1010 rep	Forward: GGAGCAGAAGAGCATACTCTGG Reverse: GGACTAGTCTGAAAGCGACCAGGTGCTCG	This study
pTrc99a Δ oriR	Forward: CGCTTACAGACAAGCTGTGACCG Reverse: GGACTAGTGTCTGACGCTCAGTGGAACGAA	This study
galP3	Forward: AGACATATGCCTGACGCTAAAAACAGGGGCGGTC Reverse: AACTGCAGGCAGAGGATAGAGCGAAGAA	This study
4162	Forward: CTAGTCTAGACGTTTCATGTCTCCTTTTTTATGTAC Reverse: CTAGGAGCTCTGCCGATATTGACTACCGGA	This study
5899	Forward: CTAGTCTAGAGCTTATGCCCATGCAACAGAAAC Reverse: CTAGGAGCTCTTAATTAGCCATTTGCTGC	This study
galPdel	Forward: GAAAATCTTCGAACTGGCGGGTTATACCAACACTAC CGAGCAAATGTGGGGTTCGAGTGTGACGGAAGATCACTTCGC Reverse: CGCGCAGTTTACGACCTTTCATCAGATTACGTTCAAT ATGTTCCAGCGAACTCCGGTAGTCAATATCGGCAGAGCTC	This study

Table 2 Comparison of fermentation profiles of succinate production from glucose and xylose by *E. coli* KJ122 and its derivatives.

Strains	Carbon sources	Sugars residual (g/L)		Succinate (g/L)	Acetate (g/L)	Maximum biomass (g/L)	Succinate yield (g succinate/sugar consumed) ^A	Maximum productivity (g/L/h) ^B
		Glucose	Xylose					
KJ122	5% xylose	-	8.50±0.37 ^e	35.36±0.14 ^l	4.35±0.10 ^h	1.93±0.12 ^{dc}	0.82±0.01 ^d	0.42±0.00 ^f
KJ122	5% glucose	0.00±0.00 ^h	-	44.17±0.78 ^j	4.09±0.78 ^h	2.03±0.04 ^{cd}	0.88±0.01 ^{abc}	0.87±0.02 ^d
KJ122	10% glucose	5.02±0.24 ^e	-	85.46±1.78 ^{ab}	8.07±0.77 ^{cd}	2.27±0.10 ^{ab}	0.88±0.02 ^{abc}	1.21±0.21 ^{abc}
KJ122	10% xylose	-	53.15±1.10 ^a	37.49±1.72 ^k	7.15±0.78 ^e	1.80±0.03 ^e	0.81±0.06 ^d	0.31±0.01 ^f
KJ122	Mixed sugars ^C	0.00±0.00 ^h	22.81±2.13 ^c	68.28±1.71 ^g	7.94±0.35 ^{cd}	2.04±0.03 ^{cd}	0.87±0.00 ^{bc}	1.11±0.03 ^{abc}
KJ122	Hydrolysate ^D	0.00±0.00 ^h	17.42±0.68 ^d	66.45±0.94 ^h	5.52±0.43 ^g	NM	0.80±0.01 ^d	0.68±0.06 ^e
AS1600a	10% xylose	-	5.00±0.28 ^{ef}	84.26±1.37 ^b	8.41±0.75 ^{ab}	2.14±0.10 ^a	0.88±0.00 ^{abc}	0.96±0.00 ^d
AS1600a	10% glucose	15.05±0.05 ^a	-	75.09±1.68 ^d	6.27±0.29 ^f	2.17±0.24 ^{bc}	0.86±0.01 ^c	0.91±0.05 ^d
AS1600a	Mixed sugars	3.24±0.36 ^f	5.03±0.57 ^f	84.23±0.46 ^b	7.90±0.93 ^{cd}	2.43±0.03 ^a	0.90±0.02 ^a	1.12±0.07 ^{abc}
AS1600a	Hydrolysate	13.53±0.84 ^b	1.21±0.45 ^g	72.66±0.59 ^e	8.33±0.31 ^{abc}	NM	0.87±0.01 ^{abc}	0.59±0.01 ^e
KJΔgalP + pLOI5747	10% xylose	-	40.48±1.09 ^b	46.32±1.69 ⁱ	4.21±0.13 ^h	2.00±0.08 ^{cd}	0.75±0.01 ^e	0.39±0.01 ^f
KJΔgalP + pLOI5747	10% glucose	6.31±0.96 ^d	-	86.88±0.93 ^a	8.42±0.37 ^{ab}	2.36±0.07 ^a	0.91±0.00 ^a	1.24±0.02 ^{ab}
KJΔgalP + pLOI5747	Mixed sugars	0.99±0.25 ^g	21.16±1.64 ^c	70.20±1.58 ^f	9.70±0.23 ^a	1.88±0.06 ^e	0.87±0.00 ^{abc}	1.10±0.02 ^c
KJΔgalP + pLOI5746	10% xylose	-	6.99±0.50 ^{ef}	85.18±0.34 ^{ab}	8.83±0.03 ^b	2.15±0.13 ^c	0.90±0.00 ^{ab}	1.11±0.06 ^{bc}
KJΔgalP + pLOI5746	10% glucose	12.39±0.57 ^c	-	78.90±0.95 ^c	7.73±0.13 ^{de}	2.38±0.12 ^b	0.88±0.01 ^{abc}	0.89±0.06 ^d
KJΔgalP + pLOI5746	Mixed sugars	3.49±0.16 ^f	4.68±0.39 ^f	85.53±0.09 ^{ab}	8.78±0.21 ^b	2.17±0.09 ^c	0.90±0.00 ^{ab}	1.25±0.07 ^a

^A The succinate yield was calculated as grams of succinate formed divided by grams of the sugar consumed.

^B The maximum succinate productivity was calculated from succinate concentration in the medium divided by the incubation time.

^C Mixed sugars are comprised of 5% xylose and 5% glucose. pLOI5747 (native *galP*), pLOI5746 (mutant *galP*), NM (not measured)

^D Sugarcane bagasse hydrolysate contained 10% of initial total sugars concentration. Arabinose and galactose in hydrolysate were completely consumed at the end of fermentation.

The superscript lowercase letters (a – l) in the same column represent a significant difference between mean values of treatments ($p \leq 0.05$). Many of these comparisons were also significant at the 99% level. The experiments were performed at least duplicate and each value expressed as the mean value ± SD.

Table 3 Comparison of strain development for succinate production from xylose, sugars mixture and hemicelluloses hydrolysate fermentation.

Strain designation	Media/mode of process	Carbon sources	Succinate (g/L)	Yield (g/g substrate)	References
<i>E. coli</i> AFP184, $\Delta ldhA$, $\Delta pf1B$ and $\Delta ptsG$	Complex medium supplemented with 0.4 g/L corn steep liquor (50% solid), dual-phase fermentation	Xylose	25.0	0.50	Andersson et al., 2007
		Xylose/glucose mixtute	27.0	0.60	
<i>E. coli</i> SD121 $\Delta ldhA$, $\Delta pf1B$, $\Delta ptsG$ and cyanoacterial <i>ppc</i> overexpression	Complex medium supplemented 20 g/L tryptone and 10 g/L yeast extract, two-stage culture	50 g/L glucose and 50 g/L xylose	58.6	0.59	Wang et al., 2011
		Corn stalk hydrolysate (44 g/L initial sugars and maintain at 10 g/L during fermentation)	57.8	0.87	
<i>E. coli</i> BA204, $\Delta ldhA$, $\Delta pf1B$, Δppc and overexpression of ATP-forming (PEPCK)	Complex medium supplemented with LB, dual-phase fermentation	20 g/L xylose	9.58	0.87	Liu et al., 2012
		10 g/L xylose +10 g/L glucose	9.18	0.72	
		Corn stalk hydrolysate (20 g/L total sugars)	11.13	1.02	
<i>E. coli</i> BA305, $\Delta ldhA$, $\Delta pf1B$, Δppc and overexpression of ATP-forming (PEPCK)	Complex medium, dual-phase fermentation.	2 g/L glucose + 13.45 g/L xylose + 2 g/L arabinose	18.88	1.10	Liu et al., 2013a
		Sugarcane bagasse hydrolysate (19.66 g/L total sugars)	19.20	0.96	
<i>E. coli</i> BA305, $\Delta ldhA$, $\Delta pf1B$, Δppc , $\Delta ptsG$ and overexpression of ATP-forming (PEPCK)	Complex medium supplemented with LB,				Liu et al., 2013b
	Simple batch fermentation	20 g/L xylose	5.2	0.72	
	Simple batch fermentation	9 g/L xylose + 9 g/L glucose	10.6	NR	
	Simple batch fermentation	Sugarcane bagasse hydrolysate	10.1	0.66	
	Feb-batch fermentation.	Sugarcane bagasse hydrolysate	39.3	0.97	
<i>E. coli</i> BA305, $\Delta ldhA$, $\Delta pf1B$, Δppc and overexpression of ATP-forming (PEPCK)	LB medium supplemented with chemically defined medium, repetitive fermentation.	Xylose	24.0	0.98	Liang et al., 2013
		Sugars mixture	29.5	0.95	
		Sugarcane bagasse hydrolysate	24.5	0.87	

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7	<i>E. coli</i> DC115, $\Delta ldhA$, $\Delta pflB$,		20 g/L xylose	12.1	0.67	
8	$\Delta ptsG$ selected by the atmospheric	LB medium supplemented with	3 g/L glucose, 2 g/L arabinose and 30			
9	and room-temperature plasma	chemically defined medium, simple	g/L xylose	27.7	0.79	Jiang et al.,
10	mutation system combining with a	bath fermentation.	Corn stalk hydrolysate (35 g/L total			2014
11	15 th serials transfer in 1.5% xylose		sugars)	21.1	0.76	
12						
13	<i>E. coli</i> BA408, $\Delta ldhA$, $\Delta pflB$, Δppc ,		24 g/L xylose + 6 g/L glucose	24.6	0.81	
14	$\Delta ptsG$ and overexpression of ATP-	LB medium supplemented with				
15	forming (PEPCK) 10 th serials	chemically defined medium, simple	Corn stalk hydrolysate (35 g/L total			Bao et al.,
16	transfer in 15 g/L sugars mixture	bath fermentation.	sugars)	23.1	0.85	2014
17						
18	<i>E. coli</i> AS1600a , $\Delta ldhA$, $\Delta adhE$,		100 g/L xylose	84.7	0.90	
19	$\Delta ackA$, $\Delta (focA-pflB)$ $\Delta mgsA$	A low salt medium (AM1, 4.2 g/L	50 g/L glucose+ 50 g/L xylose	84.2	0.88	
20	$\Delta poxB$ $\Delta tdcDE$ $\Delta citF$ $\Delta aspC$ $\Delta sfcA$	total salt), simple batch	50% (v/v) sugarcane bagasse			
21	$\Delta ptsI$, and 16 th serials transfer in	fermentation	hydrolysate + 75 g/L glucose (100 g/L	72.7	0.88	This study
22	10% xylose		total sugars)			
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Figure 1

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